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HUMAN LISTERIOSIS – GROUPING OF HUMAN  
*LISTERIA MONOCYTOGENES* ISOLATES  
WITH PFGE AND *ASCI* RESTRICTION ENZYME

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ACADEMIC DISSERTATION

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For his invisible qualities are clearly seen  
from the world's creation onward, because  
they are perceived by the things made, even  
his eternal power and Godship, so that  
they are inexcusable.

(to the Romans 1:20)

To my family

## ABSTRACT

Isolates of *Listeria monocytogenes* (N=932) collected from human cases of invasive listeriosis in Sweden between 1958 and 2010 were serotyped and characterised with pulsed-field gel electrophoresis (PFGE) and *AscI* restriction enzyme. The genotype diversity of *L. monocytogenes* isolates was investigated and related to genotypic results from epidemiological information on human infection, in order to detect possible clustering of *L. monocytogenes* genotypes over time, season, location, age, or gender (Paper I). From 1972 to 1995, serovar 4b was the predominant serovar; however, in 1996, serovar 1/2a became the major serovar among human listeriosis cases in Sweden. Based on the number and distribution of all bands in the profile, 63 PFGE types belonging to serovars 1/2b, 3b and 4b and 119 PFGE types belonging to serovars 1/2a and 1/2c were identified (Paper I). The PFGE types were further assembled into PFGE groups, based on the number and distribution of small bands below 145.5 kb (Papers II and III). As the genomic region of small bands is genetically more conservative than in large bands, the distribution of small bands establishes relatedness of strains and defines genetic markers for both lineages. Cold-smoked salmon (*Salmo salar*) and gravad salmon packed under modified atmosphere or vacuum from three manufacturers were purchased in Sweden and Germany in 2005 and the occurrence and levels of *L. monocytogenes* were analysed (Paper IV): 56 products were analysed and eleven harboured *L. monocytogenes*. From the positive samples, 56 isolates were analysed with *AscI*, and 11 isolates were further analysed with *ApaI*: five *AscI* PFGE types were identified, four belonging to serovar 1/2a and one to 4b. Forty-three (n=43: 76.8%) isolates shared serovar 1/2a and 13 (23.2%) shared serovar 4b and all *AscI* types were identified among human clinical strains in Sweden. Moreover, three gravad salmon samples harboured two PFGE types each from different lineages, serovar 1/2a and serovar 4b. Although, in most of the products, the level of *L. monocytogenes* was less than 100 cfu/g, the highest level was 1500 cfu/g. The occurrence of *L. monocytogenes* was 12.9% in gravad salmon, encountered in three manufacturers (A, B, C) and 28% in cold-smoked salmon only from manufacturer A. Although the level of *L. monocytogenes* in RTE fish products is generally low, these products, should be considered possible sources of listeriosis in Sweden. A patient may harbour more than one *L. monocytogenes* PFGE type that can be determined through PFGE and *AscI* restriction enzyme. However, to avoid misleading conclusions, several *L. monocytogenes* colonies should be isolated and characterised from different sites from the same patient or mother-baby pairs (Paper V).

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## LIST OF PAPERS

The thesis is based on the following papers, which will be referred to in the text by Roman numerals:

- I. **Lopez-Valladares, G.**, Tham, W., Parihar, V.S., Helmersson, S., Andersson, B., Ivarsson, S., Johansson, C., Ringberg, H., Tjernberg, I., Henriques-Normark, B., Danielsson-Tham, M.-L. (2014). Human isolates of *Listeria monocytogenes* in Sweden during half a century (1958-2010). *Epidemiology and Infection* 20, 1–10, PMID: 24480252.
- II. **Lopez-Valladares, G.**, Danielsson-Tham, M.-L., Goering, R., Tham, W. (2015). Division of human *Listeria monocytogenes* Pulsed-Field Gel Electrophoresis (PFGE) types belonging to lineage I (serovar 4b, 1/2b and 3b) into PFGE groups. *Foodborne Pathogens and Disease* 12, 447– 453
- III. **Lopez-Valladares, G.**, Danielsson-Tham, M.-L., Goering, R., Tham, W. (2017). Lineage II (serovar 1/2a and 1/2c) human *Listeria monocytogenes* Pulsed-Field Gel Electrophoresis types divided into PFGE groups using the band patterns below 145.5 kb. *Foodborne Pathogens and Disease* 14, 8–16
- IV. Peiris, P. I., **Lopez-Valladares, G.**, Parihar, V.S., Helmersson, S., Barbuddhe, A., Tham, W., Danielsson-Tham, M.-L. (2009). Gravad (Gravlax) and cold-smoked salmon, still a potential source of listeriosis. *Journal of Foodservice*, 20, 15–20.
- V. Tham, W., **Lopez-Valladares, G.**, Helmersson, S., Österlund, A., Danielsson-Tham, M.-L. (2007). More than one variant of *Listeria monocytogenes* isolated from each of two human cases of invasive listeriosis. *Epidemiology and Infection* 135, 854–856.

## ABBREVIATIONS

BHI	brain heart infusion
bp	base pair
BSA	bovine serum albumin
Cas	CRISPR associated
CC	clonal complex
cfu	colony forming units
CNS	central nervous system
CRISPR	clustered regularly interspaced short palindromic repeats
CSF	cerebrospinal fluid
DNA	deoxyribonucleic acid
EC	epidemic clone
ECDC	European Centre for Disease Prevention and control
EDTA	ethylenediaminetetraacetic acid
EFSA	European Food Safety Authority
ESP	N-lauroylsarcosin, EDTA, pronase
ET	electrophoretic type
ETEC	enterotoxigenic <i>Escherichia coli</i>
EU	European Union
EURL	European Union reference laboratory
G-C	guanine and cytosine
ISOPOL	International Symposium on Problems of Listeriosis
kb	kilobase
<i>L.</i>	<i>Listeria</i>
LPSN	list of prokaryotic names with standing in nomenclature
MEE	multilocus enzyme electrophoresis
MLGT	multilocus genotyping
MLST	multilocus sequence typing
MLVA	multiple-locus variable-number tandem-repeat analysis
MvLST	multi-virulence-locus sequence typing
NaCl	sodium chloride
NMKL	Nordic committee on food analysis
NRLs	National Reference Laboratories
PALCAM	polymyxin acriflavin lithium chloride ceftazidime aesculin mannitol agar
PCR	polymerase chain reaction
PEFA	4-(2-Aminoethyl)-benzenesulfonyl fluoride, hydrochloride
PFGE	pulsed-field gel electrophoresis
PI-PLC	phosphoinositide phospholipase C
RFLP	restriction fragment length polymorphism
RTE	ready-to-eat
SNP	single nucleotide polymorphism
SOP	standard operating procedures
ST	sequence typing
SLV	the National Food Administration
SVA	National Veterinary Institute

TESSy	the European Surveillance System
TBE	Trisbas, Boric acid, EDTA buffer
TE	Tris-HCl, EDTA buffer
TN	Tris-HCl, NaCl
VTEC	verotoxin-producing <i>Escherichia coli</i>
WGS	whole genome sequencing

# 1 INTRODUCTION

Listeriosis is commonly a zoonotic food-borne disease mostly transmitted through ready-to-eat (RTE) foods contaminated with *Listeria monocytogenes*, such as gravad/smoked fishes, soft cheeses and deli meat products (Novelli *et al.*, 2017). The new generation of refrigerated RTE foods, that pose most concern for listeriosis, are products normally eaten without further bactericidal treatment, with no inhibitory organic acids or lack of high counts of competing microorganisms, and with extended shelf life (Luber *et al.*, 2011). The disease listeriosis can lead to clinical manifestations, such as gastroenteritis, septicaemia, encephalitis, meningitis, and abortion (McLauchlin *et al.*, 2004). Listeriosis is one of the food-borne infections with a high fatality rate: in 2015, fatality was 12.3% of 2200 cases in EU (EFSA, 2016). *L. monocytogenes* is psychrotrophic and can grow without oxygen; therefore, food products/dishes stored in vacuum and in modified atmospheres with extended shelf life provide the opportunity for *L. monocytogenes* to multiply to large numbers towards the end of the shelf life without having to compete with most other food-borne microorganisms (Lado and Yousef, 2007). The prevalence of *L. monocytogenes* in RTE foods is generally low, and seldom exceeds the EU safety limit (< 100 cfu/g) on the day of production. At retail levels, non-compliance has been highest in fishery products and soft/semi-soft cheeses (EFSA, 2016).

In 2015, 2206 cases of invasive listeriosis were reported from 28 member states in the EU, and the incidence of listeriosis in 2014 and in 2015 was 4.6 cases per million habitants (EFSA, 2016). Elderly, pregnant women, neonates and immuno-compromised individuals are particularly susceptible to *L. monocytogenes*, although, the number of cases among the elderly population (> 64 years) increased from 56% in 2008 to 64% in 2013 (EFSA, 2016).

According to the World Health Organisation, food hygiene comprises the conditions and measures necessary to ensure the safety of food from production to consumption. In times of decreasing budgets, there is little room for preventive food hygiene. Inadequate cleaning procedures, lack of interest or knowledge, and complex equipment also increase the risk of food-borne infections. In addition, personal hygiene could be improved, such as hand cleaning, among food workers (Lindqvist *et al.*, 2000; Çakıroğlu & Uçar, 2008). Changes in lifestyle, social attitudes, and eating habits increase the opportunity for transmission of pathogenic microorganisms through contaminated foods (de Oliveira *et al.*, 2010). Since the consumption of contaminated food was identified as the main vehicle for *Listeria* infection (causing large outbreaks in Europe and USA), both the disease and *L. monocytogenes* have become a concern for food-processing manufacturers and public health authorities globally, and a health hazard and economic problem for the food industry (Giovannacci *et al.*, 1999; Stasiewicz *et al.*, 2015).

Molecular typing used to classify and compare food borne bacterial pathogens and with epidemiological investigations enable effective control and preventative measures to be implemented. Molecular typing methods are used in human listeriosis surveillance, for tracing the source of outbreaks, and for assessing the genetic diversity (generated by mutations, recombination or gene transfer) and relationships among *L. monocytogenes* isolates from one source or between various sources. Pulsed-field gel electrophoresis (PFGE) with restriction enzymes *AseI* and *ApaI* has been the gold standard molecular method for characterising *L. monocytogenes* since the middle of 1990s (Brosch *et al.*, 1996; Kérouanton *et al.*, 1998;

Cantinelli *et al.*, 2013; Michelon *et al.*, 2015; Kramarenko, *et al.*, 2016; Camargo, *et al.*, 2016). Various typing methods that analyse nucleotides within specific genes have been compared with PFGE including multiple-locus variable-number tandem-repeat analysis (MLVA), multilocus sequence typing (MLST) and multi-virulence-locus sequence typing (MvLST). Although, PFGE typing is labour-intensive, it probes the entire genome and is reproducible, whereas, MLST only analyses seven housekeeping genes. MvLST only analyses six to eight virulence genes, and MLVA analyses multiple tandem repeat sequences (Miya *et al.*, 2008; Cantinelli *et al.*, 2013; Lunestad *et al.*, 2013). In a comparison of results from PFGE, MLST and MvLST among *L. monocytogenes* clonal groups, Cantinelli *et al.* (2013) observe MvLST is no more discriminative than MLST, and PFGE has a higher discriminatory power than MLST.

PFGE characterisation of *L. monocytogenes* isolates from human patients and foods has contributed to the identification of human listeriosis outbreaks in Sweden since 1994 (Ericsson *et al.*, 1997; Danielsson-Tham *et al.*, 2004; Thisted Lambertz *et al.*, 2013). In several epidemiological investigations, a large number of PFGE types with identical DNA restriction fragments profiles have been identified among humans, animals, foods, food-processing environments, and other sources. Despite these diversifications, researchers conclude there are few prevalent *L. monocytogenes* clonal groups distributed worldwide (den Bakker *et al.*, 2010; Chenal-Francisque *et al.*, 2011; Cantinelli *et al.*, 2013; Haase *et al.*, 2014; Chen *et al.*, 2016a).

Various PFGE types have been involved in large national and multinational outbreaks and these types, called epidemic clones (EC), have been defined based on MEE, ribotyping and PFGE, and later by MvLST. Some widespread epidemic clones such as ECI and ECII, have been included in major groups through MLST and are called Clonal Complex (CC), *e.g.*, ECI was included in CC1 and ECII in CC6 (Cantinelli *et al.*, 2013). Most CC of *L. monocytogenes* have persisted over decades and only a small number of new CC have been identified in recent years (Haase *et al.*, 2014).

However, whole genome sequencing (WGS) analysis of the virulence determinants and genetic diversity (prophage, plasmids, SNP and insertion/deletion mutations) in the bacterial genome provides a greater level of discrimination for investigating *L. monocytogenes* isolates to establish epidemiologic links during surveillance, outbreak investigations, and for source tracking in Sweden and within the EU and other countries (Gilmour *et al.*, 2010; Jackson *et al.*, 2016; Kwong *et al.*, 2016). Predictions of conventional typing results (MLST, MvLST, PFGE, serotyping) are even possible *in silico* from WGS data (*in silico* typing) (Bikandi *et al.*, 2004; Chen *et al.*, 2016a; Kwong *et al.*, 2016).

The current thesis compiles information on listeriosis and characteristics (phenotypical and molecular) of *L. monocytogenes* isolates from humans and foods in Sweden and in several countries, especially within the EU. Furthermore, as all *AscI* PFGE types identified during the study period could be further assembled into PFGE groups based on small restriction fragments below 145.5 kb, a new procedure for improving the identification of a *L. monocytogenes* isolate is proposed.

## 2 REVIEW OF THE LITERATURE

### 2.1 History

Gustav Hülphers, a veterinarian at the Veterinary Institute of Stockholm, Sweden, first identified the bacterium *Listeria monocytogenes* in laboratory rabbits in 1910. In 2004, the original Swedish paper was translated into English and was published in the proceedings of “ISOPOL XV, International Symposium on Problems of Listeriosis”, Uppsala, Sweden. Hülphers described carefully the morphology, the cultural characteristics, the biochemical properties, the growth limits of the bacterium and the pathological-anatomical changes in rabbits: he called the new bacterium *Bacillus hepatitis* (Hülphers, 1911 and 2004; Nyfeldt, 1940; Gray & Killinger, 1966). In 1924, researchers from United Kingdom observed a similar bacterium, isolated from laboratory rabbits and guinea pigs at the University of Cambridge and called it *Bacterium monocytogenes* (Murray *et al.*, 1926). A researcher from South Africa (Pirie, 1927), found this pathogen in gerbils (*Iatera lobenquiae*), known as desert or African jumping mouse, and called it *Listerella hepatolytica* in honour of Lord Lister; thereafter, Pirie called the organism *Listerella monocytogenes*. In 1929, the first human case of listeriosis was reported by Nyfeldt in Denmark (Nyfeldt, 1929), where the microorganism was obtained from blood. However, in 1919, an unknown bacterium of diphtheroid type was isolated from cerebrospinal fluid (CSF) of a soldier in France and after 20 years, the organism was identified as *Listeria monocytogenes* (McLauchlin *et al.*, 1986). In Australia, cases of meningitis due to diphtheroids, possibly *Listeria*, were reported by Atkinson in 1917. In 1935, a case of meningo-encephalitis was reported in an adult, and in 1936, four cases of listeriosis in three newborn infants and an adult in USA were reported. Milk was suggested as a possible source of infection due to a similar organism being found in cows with encephalitis in 1934–1935 (Burn, 1935 and 1936). A similar organism was recovered from the brain of sheep by Gill in 1931 (Nyfeldt, 1940). Pirie changed the name of the bacterium to *Listeria monocytogenes* in 1940, as the name *Listerella* was used for other species (Pirie, 1940). One of the eminent researchers who contributed the most to the study of *L. monocytogenes* since the beginning of the 1950s was Heinz P. R. Seeliger from East Germany. Since 1955, he has published more than 500 papers (scientific papers, popular science papers and textbooks), mostly about *Listeria* (Miller *et al.*, 1990). Seeliger considered the theory of food infectious disease of substantial importance, as *Listeria* could be proved to cause epidemics (Seeliger, 1955). Due to his intensive work, he observed the first listeriosis outbreak reported in the world (1949–1957 in Halle, Germany) and suspected unpasteurised milk, sour milk, whipped cream and cottage cheese were the sources of infection in the outbreak. For this reason, Seeliger speculated contaminated food could be the route of infection by *L. monocytogenes* in humans, *i.e.* the food-borne route (Seeliger, 1961).

### 2.2 Taxonomy

*L. monocytogenes* belongs to the phylum: *Firmicutes*, class: *Bacilli*, order: *Bacillales*, family: *Listeriaceae*, and the genus *Listeria*. The genus *Listeria* comprises seventeen species and six subspecies: *L. monocytogenes*, *L. grayi* subsp. *grayi*, *L. grayi* subsp. *murrayi*, *L. innocua*, *L. seeligeri*, *L. welshimeri*, *L. ivanovii* subsp. *ivanovii*, *L. ivanovii* subsp. *londoniensis*, *L. marthii*, *L. rocourtiae*, *L. weihenstephanensis*, *L. fleischmannii* subsp. *fleischmannii*, *L. fleischmannii*

*subsp. coloradonensis*, *L. riparia*, *L. grandensis*, *L. floridensis*, *L. cornellensis*, *L. aquatica*, *L. newyorkensis*, *L. booriae* (Weller *et al.*, 2015; Orsi & Wiedmann, 2016; LPSN, 2017). Six species belonging to *Listeria sensu strictu* (*L. monocytogenes*, *L. marthii*, *L. innocua*, *L. welshimeri*, *L. ivanovii*, *L. seeligeri*) are ubiquitous and commonly found in diverse environments, according to phenotypic characteristics such as motility and growth at 4°C. The remaining 11 species belong to *Listeria sensu lato*. There are proposals (Orsi & Wiedmann, 2016) for dividing and reclassifying the genus *Listeria* (17 species) into four genera: genus *Listeria* (including the six *Listeria sensu strictu*), *Murraya* (*L. grayi*), *Mesolisteria* (*L. fleischmannii*, *L. floridensis* and *L. aquatica*), and *Paenilisteria* (the remaining 7 species). The last three genera are not pathogenic; among those, only *L. grayi* is motile and positive for the Voges-Proskauer test, and only *L. floridensis* is unable to reduce nitrate (Weller *et al.*, 2015; Orsi & Wiedmann, 2016).

## 2.3 Morphology

Morphologically, *Listeria* spp. are rod-shaped with rounded ends, vary in size between 0.4 and 0.5 µm in diameter and 1 and 2 µm in length, and do not form spores or capsules. Characteristically, young cultures are Gram-positive but may become Gram-negative as they mature. *Listeria* colonies grow on nutrient agar after 24 hours at 20–25°C and are round, 0.5–1.5 mm in diameter, translucent, low convex with a glistening and smooth surface (S-forms). After 3–7 days of incubation, the colonies are 3–5 mm in diameter with rough surface (R-forms). All species belonging to *Listeria sensu strictu* and *L. grayi* are motile due to peritrichous flagella especially when grown in liquid culture at 10° – 25°C (Seeliger & Jones; 1986, Farber & Peterkin, 1991; Weller *et al.*, 2015).

## 2.4 Growth and biochemical properties

The growth range for *Listeria* bacteria extends from pH 4.4 to pH 9.5 at temperatures between 0.4°C and 52°C, in food with up to 14% NaCl and at a water activity ( $a_w$ ) as low as 0.92. The metabolism is aerobic and facultative anaerobic (Seeliger & Jones, 1986; Farber & Peterkin, 1991; Swaminathan *et al.*, 2007; Velge & Roche, 2010; Carpentier *et al.*, 2011; Iannetti *et al.*, 2016; Wemmenhove *et al.*, 2016). Only *L. monocytogenes*, *L. ivanovii*, *L. seeligeri* and a few *L. innocua* strains are positive for haemolysis (Orsi & Wiedmann, 2016). All species are able to produce the antioxidant enzyme catalase, hydrolyse esculin and produce acid from *N*-acetylglucosamine, amygdalin, arbutin, salicin, cellobiose, *D*-fructose, and *D*-mannose. However, they are not able to reduce nitrite and produce acid from *D*-arabinose, *D*-adonitol, methyl β-*D*-xylopyranoside, raffinose, glycogen, *L*-fucose, potassium gluconate, potassium 2-ketogluconate and potassium 5-ketogluconate (Weller *et al.*, 2015). The biochemical characteristics of some of the *Listeria* species are presented in Table 1.



Table 1. Biochemical properties of some *Listeria* species (Seeliger & Jones, 1986; Leclercq *et al.*, 2010; Weller *et al.*, 2015; Orsi & Wiedmann, 2016)

Biochemical properties	<i>L. monocytogenes</i>	<i>L. innocua</i>	<i>L. ivanovii</i>	<i>L. grayi</i>	<i>L. floridensis</i>	<i>L. aquatica</i>	<i>L. rocourtiae</i>	<i>L. booriae</i>
β- haemolysis	+	-	+	-	-	-	-	-
Nitrate reduction	-	-	-	+/-	-	+	+	+
Motility	+	+	+	+	-	-	-	-
Arylamidase	-	+	+/-	+	-	-	-	-
PI-PLC	+	-	+	-	-	-	-	-
α-Mannosidase	+	+	-	+/-	-	+	+	+
D-Mannitol	-	-	-	+	-	-	+	+
D-Arabitol	+	+	+	+	-	-	-	+
D-Xylose	-	-	+	-	+	+	+	+
L-Rhamnose	+	+/-	-	-	+	+	+	+
α Methyl -D- glucoside	+	+	+	+	+	-	+	+
D-Ribose	-	-	+	+	-	+	+	+/-
Glucose-1- phosphate	-	-	+/-	-	-	-	-	-
D-Tagatose	-	-	-	-	-	+	-	-
Voges-Proskauer	+	+	+	+	-	+/-	-	-

PI-PLC, phosphoinositide phospholipase C. - = negative reaction. + = positive reaction

## 2.5 Habitat

*Listeria* spp. are widely distributed in the environment and can be isolated from a variety of sources such as soil, surface, rivers, canal waters, sewage, decaying vegetation (Beuchat, 1996) and from the surfaces of equipment, floors and wall of the food processing plants (Franco-Abuín, *et al.*, 1996; Unnerstad *et al.*, 1996). *Listeria* spp. have also been isolated from faeces of domestic and wild animals, birds, tics, larvae, ensilage, processed foods (ready-to-eat food, food requiring cooking or reheating) and different kinds of raw foods such as vegetables, fruits, milk, cheese, meat, fish and crustaceans (Gray, 1963; Loncarevic *et al.*, 1996a; Loncarevic *et al.* 1996b).

## 2.6 Phenotypic typing methods

Serotyping is the standard subtyping method for analysing phenotypic characteristics of *Listeria* and is widely used in epidemiological surveillance of human and food isolates. Furthermore, the method has the advantage that results can be compared between different laboratories. Although conventional serotyping by agglutination is traditionally used, molecular serotyping based on specific genes has been developed (Seeliger & Höhne, 1979; Bannerman, 1995; Borucki & Call, 2003; Doumith *et al.*, 2004; Nightingale *et al.*, 2007; Kérouanton *et al.*, 2009).

### 2.6.1 Conventional serotyping

Serological analysis of somatic and flagellar antigens is the common technique for characterisation of different genus *Listeria* isolates. There are 15 somatic (I–XV) and 5 flagellar antigenic factors (A–E). The species *L. monocytogenes* is divided based on 13 somatic antigenic factors (O-factor) into four serogroups (1/2, 3, 4, 7). Serogroups 1/2 and 3 are further divided into 6 serovars (1/2a, 1/2b, 1/2c, 3a, 3b, 3c), based on four flagellar antigenic factors (H-factor). Although, the remaining serogroups 4 and 7 share the same flagellar factors (ABC), the somatic antigenic factors further divide them in serovars: 4a, 4ab, 4b, 4c, 4d, 4e and 7. Thus, the species *L. monocytogenes* are divided into 13 serovars (Table 2) (Seeliger & Höhne, 1979).

### 2.6.2 Molecular serotyping

Polymerase chain reaction (PCR) serotyping is a molecular method based on the amplification of specific regions in the bacterial genome. Two PCR assays with seven different marker genes are used. These PCR assays are based on the detection of serogroup specific regions leading to five molecular serogroups IIa, IIb, IIc, IVa and IVb. The first PCR assay detects the presence of six genes: *prfA* (specific for *L. monocytogenes*), *prs* (specific for the genus *Listeria*), *orf2819* (specific for molecular serogroup IIb and IVb), *orf2110* (specific for molecular serogroup IVb), *lmo0737* and *lmo1118* (specific for molecular serogroups IIa and IIc). The second PCR detects the *flaA* gene specific for molecular serogroups IIa and IVa (Table 2). However, the combination of sigma factor *sigB* gene, which is involved in the stress response regulation of *L. monocytogenes* (Severino *et al.*, 2007), with multiplex PCR increases discrimination of atypical serovar 4b. Unfortunately, PCR serotyping is unable to distinguish all serovars, as with conventional serotyping. However, PCR serotyping distinguishes the most common serovars involved in human listeriosis (Borucki & Call, 2003; Doumith *et al.*, 2004; Nightingale *et al.*, 2007; K  rouanton *et al.*, 2009).

Table 2. Comparison of conventional and molecular serotyping

Molecular serogroup	IIa	IIc	IIb	IVa	IVb
Serovar	1/2a, 3a	1/2c, 3c	1/2b, 3b, 7	4a, 4c	4b, 4ab, 4d, 4e
PCR I	<i>lmo1118</i>	<i>lmo1118</i>	-	-	<i>orf2110</i>
	<i>lmo0737</i>	<i>lmo0737</i>	<i>orf2819</i>	-	<i>orf2819</i>
	<i>prs</i>	<i>prs</i>	<i>prs</i>	<i>prs</i>	<i>prs</i>
	<i>prfA</i>	<i>prfA</i>	<i>prfA</i>	<i>prfA</i>	<i>prfA</i>
PCR II	<i>flaA</i>	-	-	<i>flaA</i>	-
Serovar	1/2a	1/2c	1/2b	4a	4b
O-factor	I/II, I	I/II, I	I/II, I	V, VII, IX	V, VI
H-factor	AB	BD	ABC	ABC	ABC
Serovar	3a	3c	3b	4c	4ab
O-factor	I/II, IV	I/II, IV, (XII), (XIII)	I/II, IV, (XII), (XIII)	V, VII	V, VI, VII, IX, X
H-factor	AB	BD	ABC	ABC	ABC
Serovar	nd	nd	7	nd	4d
O-factor	nd	nd	XII, XIII	nd	V, VI, VIII
H-factor	nd	nd	ABC	nd	ABC
Serovar	nd	nd	nd	nd	4e
O-factor	nd	nd	nd	nd	V, VI, (VIII), (IX)
H-factor	nd	nd	nd	nd	ABC

- = gene is absent. nd= no data

## 2.7 The genome of *Listeria monocytogenes*

The genome of *L. monocytogenes* is a circular chromosome with a size varying between 2.7 and 3.0 Mb (Hain *et al.*, 2007; Bécavin *et al.*, 2014). The nitrogenous bases of a DNA molecule are bound by hydrogen bonds and are either Guanine or Cytosine (G-C) and Adenine or Thymine (A-T). The G-C pair is bound by three bonds, whereas, A-T pairs are connected by two bonds. The G-C base pairs are more stable under higher temperatures than A-T base pairs, although the G-C content does not play a role in the stability of DNA. The percentage of these base pairs may refer to whole genome or a specific fragment of DNA or RNA, *i.e.* a fragment of the genetic material that is part of a single gene, gene clusters, or even a non-coding region. The genomic G-C content of *L. monocytogenes* is low, between 36–39% (Brosch *et al.*, 1991; Yakovchuk *et al.*, 2006; Hain *et al.*, 2007; Zheng & Wu, 2010; Bécavin *et al.*, 2014). In *Listeria* PFGE, the restriction enzymes with recognition sequences containing G and C nucleotides are used to avoid multiple bands in bacterial PFGE profiles. Among the different *L. monocytogenes* serovars, the genomes are highly syntenic: display a similar size, G-C content, percentage of protein coding DNA and average length of protein coding genes (Kuenne *et al.*, 2013), where surface proteins display the highest number of single nucleotide polymorphisms (SNP) (Hain *et al.*, 2012).

The species *L. monocytogenes* have a highly conserved but open pan-genome permitting limited integration of foreign chromosomal DNA (called mobile genetic elements), such as bacteriophages, plasmids, transposons, genomic islands and insertion sequences, which contribute to genome diversity through mutation, recombination and duplication (Nelson *et al.*, 2004; den Bakker *et al.*, 2010; Kuenne *et al.*, 2013). *L. monocytogenes* genomes contain some accessory genes located in a highly variable chromosomal region or hyper variable hotspots (Kuenne *et al.*, 2013).

### 2.7.1 Extrachromosomal DNA in *L. monocytogenes* genome

The presence of extrachromosomal DNA is common in *L. monocytogenes* genome. The *Listeria* genomes harbour at least one prophage or part of bacteriophage genomes. The species *L. monocytogenes* has a cryptic prophage region in a single locus called monocin *lma* locus, but the gene content in the *lma* operon vary in different genomes of *L. monocytogenes* (Hain *et al.*, 2012). Comparative analysis of genome sequences belonging to serovars 4b or 1/2a strains reveal 4b strains contain fewer prophages than 1/2a strains, and are inserted into different chromosomal loci adjacent to tRNA genes (Hain *et al.*, 2006 and 2012; Kuenne *et al.*, 2013). The plasmids of genus *Listeria* are related to plasmids in *Bacillus*, *Enterococcus* and *Streptococcus* and contain a large number of mobile genetic elements (Kuenne *et al.*, 2010). Although, the *L. monocytogenes* genomes rarely harbour plasmids, several plasmid genes are frequently involved in heavy metal resistance (cadmium and arsenite resistance operons), benzalkonium chloride resistance, oxidative stress response and multidrug efflux (Lebrun *et al.*, 1992; Hadorn *et al.*, 1993; McLauchlin, 1996c; Harvey & Gilmour, 2001; Elhanafi *et al.*, 2010; Kuenne *et al.*, 2010). The presence of plasmids in *L. monocytogenes* serovar 4b isolates is lower than in serogroup 1/2 isolates (Lebrun *et al.*, 1992; Peterkin *et al.*, 1992). The *L. monocytogenes* genome contains CRISPR (clustered regularly interspaced short palindromic repeats) and protein coding genes Cas (CRISPR associated), which is an adaptive immune system to protect the bacteria against bacteriophages. Prophages and plasmid content in the bacteria can be associated with acquisition or loss events occurring during human infection, in processing plants, environment or during passage of isolates in a medium containing acriflavine, a plasmid curing agent (Margolles *et al.*, 1998b; Orsi *et al.*, 2008c; Chen *et al.*, 2016b; Chen *et al.*, 2017a). Loss or gain of prophages in the bacterial genome appears to occur promptly and frequently (Orsi *et al.*, 2008a). The variation in genome size can be attributed to the complete or partial absence of prophages and plasmid in the genome. The diversification of prophages in the species *L. monocytogenes* signifies a fundamental mechanism for short-term genome development (Orsi *et al.*, 2008c).

### 2.7.2 Virulence factors

*L. monocytogenes* is able to invade, survive and proliferate within phagocytes (macrophages) and in different tissues of several eukaryotic, non-phagocytic cells such as epithelial, endothelial, neurons and hepatocytes. Specific virulence factors provide this ability and are crucial for causing an infection in the host (Mackaness, 1962; Chakraborty *et al.*, 2000; Vázquez-Boland *et al.*, 2001a). The bacteria induce their own uptake into non-phagocytic cells. During the adhesion

and internalisation process of the bacterium into the cell, several genes such as *inlA*, *inlB*, *inlC* are involved and these are regulated by the transcriptional activator PrfA. The *L. monocytogenes* internalin genes (*inlA*, *inlB* and *inlC*) encode the surface proteins internalin A, B, C (InlA, InlB and InlC), which are associated with the attachment and invasion of the host cells. The gene for invasion-associated protein (*iap*) encodes the extracellular protein p60, a hydrolase essential for bacterial metabolism. The surface proteins Ami (an autolysin) and Lap contribute to the adhesion of *L. monocytogenes* to eukaryotic cells.

Six virulence factors (*prfA*, *plcA*, *hly*, *mpi*, *actA* and *plcB*) are involved in the intracellular parasitic life cycle of *L. monocytogenes*. In *Listeria*, these virulence genes, located in a central virulence gene cluster physically linked to a 9 kb chromosomal island, are organised into discrete genetic units known as pathogenicity islands (PAIs) or LIPI-1. The positive regulatory factor A (*prfA*) codes for the PrfA protein, a thermo-regulated transcriptional activator that activates the expression of all six genes in the cluster. In *Listeria*, virulence genes have maximal expression at 37°C (body temperature) but are weak below 30°C. Two different phospholipase C are involved in *L. monocytogenes* invasion and spread to host. The first bacterial gene phospholipase C (*plcA*) encodes the enzyme phosphatidylinositol-specific phospholipase C (PlcA), also known as PI-PLC, and participates in the rupture of the phagocytic vacuole, the primary phagosome of cells. The haemolysin gene (*hly*) encodes the haemolytic toxin protein called listeriolysin O (Hly or LLO) in *L. monocytogenes*, where its cytolytic activity is maximised at pH 5.5. The haemolytic toxin in combination with PlcA lyses the primary phagosome.

After the bacterium escapes into the cytoplasm, it multiplies intracellular. In this step, the gene actin A (*actA*) produces the surface protein actin A (ActA) responsible for motility and cell-to-cell spread. The bacterium is covered with a layer of actin filaments that subsequently rearrange into a tail to provide motility. Through movement, the organism makes contact with the plasma membrane and induces pseudopod-like protrusions that reach the adjacent cell: the organism is ingested by a double plasma membrane. The second bacterial gene phospholipase C (*plcB*) produces the enzyme phosphatidylcholine-specific phospholipase C (PlcB), a zinc-metallo-enzyme. In the adjacent cell, the PlcB enzyme in combination with Hly lyses the two-membrane phagocytic vacuole, the secondary phagosome, which surrounds the bacterium during transfer; thus, a new intracellular parasitic life cycle reinitiates. The gene *mpi* encodes the metallo-protease enzyme (Mpi), a zinc-dependent metalloprotease that processes extracellular PlcB enzyme to its mature form and contributes to dissemination of the bacterium from cell to cell (Chakraborty *et al.*, 2000; Vásquez-Boland *et al.*, 2001a; Vásquez-Boland *et al.*, 2001b; Alvarez & Agaisse, 2016).

### 2.7.3 Phylogeny of *L. monocytogenes*

Epidemiological studies use methods for determining the genetic diversity and relationships among *L. monocytogenes* isolates recovered from different sources and geographic locations. Two primary phylogenetic divisions within the species *L. monocytogenes* were first identified at the end of 1980s by multilocus enzyme electrophoresis (MEE). Among electrophoretic types (ET), division I consists of serovars 1/2b, 3b, 4a, 4b and division II serovars 1/2a, 1/2c (Bibb *et al.*, 1989; Piffaretti *et al.*, 1989). In the 1990s, new molecular subtyping methods consistently

divided *L. monocytogenes* into two corresponding groups (divisions I and II). Nucleotide sequence of the listeriolysin gene (*hly*) coincides with flagellar antigens and groups the isolates into sequence type I (1/2b, 4b) and sequence type II (1/2a, 1/2c) (Rasmussen *et al.*, 1991). MEE and ribotyping analysis divided serovars 1/2b, 3b, 4b, 4ab and serovars 1/2a, 1/2c, 3a into two subgroups (Graves *et al.*, 1994). PFGE analysis with restriction enzyme *AscI* divided isolates into two genomic divisions (division I: serovars 1/2a, 1/2c, 3a, 3c and division II: serovars 1/2b, 3b, 4b, 4d, 4e) that correlate with the flagellar (H) antigen. Furthermore, restriction enzyme *Apal* recognises two clusters in each genomic division *i.e.* cluster IA (serovars 1/2c, 3c), cluster IB (serovars 1/2a, 3a), cluster IIA (serovars 1/2b, 3b) and cluster IIB (serovars 4b, 4d, 4e) (Brosch *et al.*, 1994).

Rasmussen *et al.* (1995) analysed several *L. monocytogenes* isolates from different sources and countries and distinguished three evolutionary lines through nucleotide sequences of *flaA* (flagellin), *iap* (invasive associated protein), *hly* (listeriolysin O) and 23S rRNA genes. The grouping correlated with the serovars: the first sequence type included serovars 1/2b, 4b, the second sequence type 1/2a, 1/2c, 3 and the third serovar 4a. In addition, Wiedmann *et al.* (1997) define three distinct genetic lineages, these combine ribotype patterns and PCR-restriction fragment length polymorphism (RFLP) types of three virulence genes (*hly*, *actA*, *inlA*). All three lineages are designated in accordance with Rasmussen *et al.* (1995), *e.g.* sequence type 1 corresponds to lineage I, sequence type 2 corresponds to lineage II, and sequence type 3 corresponds to lineage III.

Nadon *et al.*, (2001) describe the relationships between *L. monocytogenes* serotypes, ribotypes and genetic lineages. Lineage I contains serotypes 1/2b, 3b, 3c, 4b, lineage II contains serotypes 1/2a, 1/2c, 3a and lineage III contains serotypes 4a, 4c. Thereafter, the designation “lineage” has been used by other researchers and remains unchanged. Multilocus sequence typing (MLST: developed by Salcedo *et al.*, 2003) for *L. monocytogenes* uses 7–9 housekeeping genes and divides the species into two genetic divisions, according to Brosch *et al.* (1994). In the 2000s, lineage III was divided into multiple distinct subgroups by sequence analyses of one or more bacterial genes. Some published studies use RFLP, nucleotide sequences of *sigB* encoding the stress response sigma factor sigB, *prfA* encoding a virulence regulator, MLST and PCR/Southern hybridisation (Tran & Kathariou, 2002; Moorhead *et al.*, 2003; Meinersmann *et al.*, 2004; Ward *et al.*, 2004; Liu *et al.*, 2006a). Sequence analyses of *sigB* and *actA* identify three subgroups in lineage III (IIIA, IIIB and IIIC) that include serovars 4a, atypical 4b, and 4c. However, no association between subgroups and serovars has been found and “these three subgroups may represent separate evolutionary lineages” (Roberts *et al.*, 2006). With a sequence of five genes (*cheA*, *phoP*, *lmo0693*, *flaR* and *lmo2537*), Orsi *et al.* (2008b) observed *L. monocytogenes* is divided into four clusters, with the fourth cluster containing the lineage III subgroup IIIB, which forms an “independent cluster”. With the use of multilocus genotyping (MLGT), Ward *et al.* (2008) identified lineage III subgroup IIIB as a fourth lineage of *L. monocytogenes* (lineage IV) which contains serotypes 4a, atypical 4b and 4c (Table 3). Lineage I appears to be almost clonal whereas, considerable horizontal gene transfer or recombination occurs in other lineages. The lineages most frequently involved in human listeriosis are lineage I (serovars 1/2b and 4b) and lineage II (serovars 1/2a and 1/2c), whereas, lineages III and IV are usually isolated from animals, food and the environment (Meinersmann *et al.*, 2004; Ward *et al.*, 2004; Liu *et al.*, 2006b; Orsi *et al.*, 2008a, b; Orsi *et al.*, 2011).

Table 3. Identification of *Listeria monocytogenes* phylogenetic lineages.

Reference	Method	Lineage I (Serovar)	Lineage II (Serovar)	Lineage III (Serovar)	Lineage IV (Serovar)
Piffaretti <i>et al.</i> , 1989	MEE	Division I (1/2b, 4a, 4b)	Division II (1/2a, 1/2c)	nd	nd
Bibb <i>et al.</i> , 1989	MEE	Cluster II (1/2b, 3b, 4b)	Cluster I (1/2a)	nd	nd
Rasmussen <i>et al.</i> , 1991	Gene sequence ( <i>lisA</i> or <i>hly</i> )	Sequence type I (1/2b, 4b)	Sequence type II (1/2a, 1/2c, 3)	nd	nd
Graves <i>et al.</i> , 1994	Ribotyping/ MEE	Ribotype $\beta$ (1/2b, 3b, 4b, 4ab)	Ribotype $\alpha$ (1/2a, 1/2c, 3a)	nd	nd
Brosch <i>et al.</i> , 1994	PFGE ( <i>Ascl</i> , <i>Apal</i> )	Division II (1/2b, 3b, 4b, 4d, 4e)	Division I (1/2a, 1/2c, 3a, 3c)	nd	nd
Rasmussen <i>et al.</i> , 1995	Gene sequence ( <i>flaA</i> , <i>iap</i> , <i>hly</i> , 23S rRNA)	Sequence type I (1/2b, 4b)	Sequence type II (1/2a, 1/2c, 3)	Sequence type III (4a)	nd
Wiedmann <i>et al.</i> , 1997	Ribotyping/ RFLP ( <i>hly</i> , <i>actA</i> , <i>inlA</i> )	Lineage I (1/2b, 4b)	Lineage II (1/2a, 1/2c, 3)	Lineage III (4a)	nd
Nadon <i>et al.</i> , 2001	Ribotyping	Lineage I (1/2b, 4b, 3b, 3c)	Lineage II (1/2a, 1/2c, 3a)	Lineage III (4a, 4c)	nd
Salcedo <i>et al.</i> , 2003	MLST (7–9 housekeeping genes)	Division II (1/2b, 4b)	Division I (1/2a)	nd	nd
Moorhead <i>et al.</i> , 2003	Gene sequence ( <i>sigB</i> /SNP)	Lineage I (1/2b, 4b)	Lineage II (1/2a, 1/2c)	Lineage III (4a, 4c and atypical 4b, 4c)	nd
Meinersmann <i>et al.</i> , 2004	MLST	Lineage I (1/2b, 3b, 4b, 4e)	Lineage II (1/2a, 1/2c, 3a)	Lineage III (4a, 4b, 4c)	nd
Ward <i>et al.</i> , 2004	Gene sequence ( <i>prfA</i> )	(1/2b, 3b, 4b, 4d, 4e)	(1/2a, 1/2c, 3a, 3c)	(4b, 4c)	nd
Roberts <i>et al.</i> , 2006	Gene sequence ( <i>sigB</i> , <i>actA</i> )	nd	nd	Subgroups IIIA, IIIB, IIIC	nd
Liu <i>et al.</i> , 2006b	PCR and Southern blott	(1/2b, 4b)	(1/2a, 1/2c)	Subgroups IIIA, IIIB, IIIC	nd
Orsi <i>et al.</i> , 2008b	Gene sequence ( <i>cheA</i> , <i>phoP</i> , <i>lmo</i> 0693, <i>flaR</i> , <i>lmo</i> 2537)	(4b)	(1/2a)	Subgroups IIIA/C and IIIB	nd
Ward <i>et al.</i> , 2008	MLGT	nd	nd	(4a, 4c, atypical 4b)	(4a, atypical 4b and 4c)

nd = no data

## 2.7.4 PFGE fragments

Pulsed-field gel electrophoresis (PFGE) is able to estimate the whole genome size of a microorganism, *i.e.* the number of base pairs contained in the genomic DNA, through the measurement of restriction fragments (Hielm *et al.*, 1998; Alduina & Pisciotta, 2015). These fragments are generated by restriction enzymes that cleave the DNA at different cleavage sites (Wilhelm *et al.*, 2003). As the genomic G-C content of *L. monocytogenes* is low, restriction enzymes with recognition sequences containing G and C nucleotides are used to avoid generating multiple bands. Suitable rare cutting restriction enzymes for *L. monocytogenes* PFGE analyses are *AscI* and *ApaI*. Usually, a PFGE type (pulsotype or pulsovar) is established based on the number and distribution of all fragments within a DNA restriction profile. *AscI* PFGE profiles of *L. monocytogenes* have two well-defined regions above and below the size of 145.5 kb. Several *L. monocytogenes* *AscI* PFGE profiles from isolates identified in outbreaks, and even among isolates from different sources, time and location, display PFGE types that differ in the region above 145.5 kb, but are identical in the region below 145.5 kb (Brosch *et al.*, 1994; Cantinelli *et al.*, 2013). The large restriction fragments usually harbour prophages and the insertion or deletion of these prophages change the number and distribution of these fragments in the upper region, generating new variants or *AscI* PFGE profiles of *L. monocytogenes* strains (Stasiewicz *et al.*, 2015; Chen *et al.*, 2017b; Li *et al.*, 2017). Among highly related *L. monocytogenes* strains the *AscI* PFGE profiles differ by only two fragments in the upper region and are similar in the lower region (Kathariou *et al.*, 2006; Gilmour *et al.*, 2010; Chen *et al.*, 2016b; Kvistholm Jensen *et al.*, 2016). These observations suggest *AscI* restriction fragments below 145.5 are more conserved than larger fragments, supporting the utility of smaller fragments in grouping *L. monocytogenes*. Furthermore, there is a correlation between *L. monocytogenes* PFGE types and serovars in both lineages I and II. However, a few *L. monocytogenes* isolates with indistinguishable PFGE profiles displaying different serovars (1/2a and 3a or 1/2b and 3b) are reported (Brosch *et al.*, 1994; Nadon *et al.*, 2001; Lukinmaa *et al.*, 2003; Revazishvili *et al.*, 2004; Gianfranceschi *et al.*, 2009).

### PFGE in silico

Analysis *in silico* “is conducted via computer simulations with models closely reflecting the real world” (Apache Software Foundation, 2016). By selecting a specific strain of *L. monocytogenes* from the available whole genome sequences (<http://insilico.ehu.es>), it is possible to make a theoretical cleavage (*in silico*) by choosing a required restriction enzyme, *e.g.*, *AscI*. Bikandi *et al.*, (2004) analysed *in silico* with PFGE and restriction enzyme *AscI* some sequenced *L. monocytogenes* strains belonging to lineage II.

## 2.8 Listeriosis in humans

Although listeriosis is considered a rare disease in humans, veterinarians have long observed listeriosis in animals. Increased knowledge of human listeriosis, improved bacteriological diagnoses, and an increasing number of bacteriological routine tests may partly explain the increase in the number of diagnosed cases of listeriosis (Larsson, 1960; Sepp & Roy, 1963; Gerdin, 1981; McLauchlin, 1996b). In addition, changes in food habits may contribute to the increase in human listeriosis cases (Allerberger & Wagner, 2010; de Oliveira *et al.*, 2010).



Modern food technology, *i.e.* the use of vacuum packaging, has led to increased opportunities for *L. monocytogenes* to multiply in RTE foods (Bērzinš *et al.*, 2007). Moreover, the susceptible human population is ever increasing, people are living longer and more people have underlying health conditions which themselves or the treatment of them increases susceptibility to listeriosis infection (Allerberger & Wagner, 2010; Todd & Notermans, 2011).

The *Listeria* species pathogenic to humans and animals are *L. monocytogenes*, *L. ivanovii* and *L. seeligeri*, although, *L. monocytogenes* is associated with the vast majority of cases of listeria infection. Listeriosis in humans manifests primarily as septicaemia, meningitis, encephalitis, gastrointestinal infection and abortion. Other less common manifestations are endocarditis, pericarditis, myocarditis, arteritis, pneumonia, sinusitis, conjunctivitis, ophthalmitis, otitis, joint infection and skin infection (Seeliger & Jones, 1986; Radostits *et al.*, 1994; Vázquez-Boland *et al.*, 2001b). Incubation times range from one day to three months (Linnan *et al.*, 1988). Goulet *et al.*, (2013) report four different median incubation periods related to clinical manifestations: 24 hours (range 6–24 h) for gastrointestinal forms; 2 days (range 1–12 days) for bacteraemia; 9 days (range 1–14 days) for central nervous system (CNS) cases; and, 27.5 days (range 17–67 days) for pregnancy-related cases.

The disease occurs sporadically and in outbreaks involving few or substantial numbers of affected individuals. However, most human cases are sporadic and thus, it is difficult to trace the implicated source. Although the incidence of listeriosis is lower than for other bacterial diseases, the fatality rate is between 20% and 40% in humans (Hof *et al.*, 1994; McLauchlin, 1996a; Magalhães *et al.*, 2015). Fatality rate is highest in susceptible populations with underlying immunosuppressive conditions, such as pregnant women, neonates and the elderly (Schlech *et al.*, 1983; Linnan *et al.*, 1988; McLauchlin *et al.*, 1991; Büla *et al.*, 1995).

Clinically healthy and convalescent humans can be carriers of *L. monocytogenes*. Positive samples have been obtained from faeces of slaughterhouse and laboratory workers and even from office workers that have no contact with listerial material. *L. monocytogenes* has been isolated from 1.2% individuals in Denmark, 2% in West Germany, 12 to 77% in The Netherlands, 5.4% in France and 0.6% in England. Pharyngeal and vaginal carriage is also reported (Bojsen-Møller, 1964; Kampelmacher & van Noorle Jansen, 1972; Carbonnelle *et al.*, 1978; Kampelmacher & van Noorle Jansen, 1979; McLauchlin *et al.*, 1986). Domestic ruminants can shed the organism in faeces or milk and the percentage of carriers among cattle is *e.g.* 52% in Denmark, 6.7% in Finland, and 6% in Sweden (Skovgaard & Morgen, 1988; Hsu *et al.*, 1990; Unnerstad *et al.*, 2000). In the pathogenesis of listeriosis, certain factors play an important role, such as age (neonates and elderly) and deficient immune status due to underlying illness (Nieman & Lorber, 1980; McLauchlin, 1990a and 1990b). *In vitro*, the pathogenic species of *Listeria* are susceptible to many common antibiotics such as penicillin, ampicillin, tetracycline and erythromycin. However, treatment *in vivo* is complicated because the bacteria grow and multiply intracellular, where antibiotics have to penetrate through the cell walls (Bannister, 1987; Nichterlein & Hof, 1991). Although ampicillin and gentamicin are still the drugs of choice for treating listeriosis, aminoglycosides, such as gentamicin, may be harmful to patients with renal failure (Mitjà *et al.*, 2009).

### 2.8.1 Human listeriosis in Europe

Several countries report an increasing tendency of human listeriosis since the 1990s. In the European Union, the incidence of listeriosis increased 30% from 2013 to 2014 (Mammina *et al.*, 2013; Iannetti *et al.*, 2016).

#### *United Kingdom*

Between 1967 and 1982, there were less than 100 human cases of listeriosis annually in England, Wales and Northern Ireland. Since the 1960s, the Public health laboratory service of London, UK, has monitored human listeriosis (McLauchlin *et al.*, 1991). From 1967 to 1985, 786 cases were reported in Britain and of 722 viable *L. monocytogenes* human isolates, 248 (34%) were associated with pregnancy, with a 36% fatality rate. Among 474 (66%) non-pregnant cases, 58% were male and 42% female. The fatality rate was higher among non-pregnant cases (44%), with the highest fatality rate being among patients over 60 years old with underlying diseases (54%). Among all 722 isolates, 423 (59%) isolates shared serovar 4b, 130 (18%) shared serovar 1/2a, 99 (14%) shared serovar 1/2b, and 29 (4%) shared serovar 1/2c (McLauchlin, 1987, 1990a and 1990b). In France, during the same period, overall fatality rate was 34% among pregnancy-associated cases and 47% among non-pregnant cases (Humbert *et al.*, 1977a & 1977b).

In Britain between 1976 and 1979, a *L. monocytogenes* serovar 4b strain belonging to a particular phage type, called “Liverpool type”, was isolated from several human patients, which meant a probable common source outbreak (McLauchlin *et al.*, 1986). In 1985, in England, Wales and Northern Ireland, the number of reported human cases was 149, and the cases increased considerably between 1987 and 1989 to more than 250 cases annually (McLauchlin *et al.*, 1991). At the end of 1987, the Department of Health in London, UK, issued a hazard warning for contaminated soft Swiss cheeses after the Swiss outbreak of 1983–1987. In February 1989, the government reiterated the warning on soft cheeses to vulnerable groups, and in July 1989, issued a warning on the consumption of pâté (McLauchlin *et al.*, 1988 and 1991). The majority of *L. monocytogenes* human isolates from 1987 belonged to serovar 4b (71%) and 42 people died among 130 non-pregnant cases (McLauchlin *et al.*, 1988).

Between 1967 and 1988 in Scotland, 220 cases of listeriosis were reported, with fetomaternal cases frequently involved (198 cases). Among the fetomaternal cases, the rate increased from 38 (1977–1981) to 72 cases (1987–1988). *L. monocytogenes* serovar 4b was predominant, especially in the last two years (63%). The incidence in Scotland subsequently increased from 0.5 cases per million habitants in 1967–1971 to 0.6 (1972–1976), 1.5 (1977–1981), 2.2 (1982–1986), and 7.0 cases per million habitants (1987–1988) (Campbell, 1990). During the period 1983 to 1994 in England, Wales and Northern Ireland, 1844 cases of listeriosis were reported, of which 655 (36%) of cases were associated with pregnancy. Among 1620 available *L. monocytogenes* human isolates from this period, serovar 4b accounted for 39% to 72% of cases annually, and at least 57% among pregnancy-associated cases. Among non-pregnancy associated cases, serovar 4b accounted for 47% to 64% of cases; however, the serovar became less common in 1993 (34%) and 1994 (24%). Instead, serovar 1/2a became more dominant in 1993 (37%) and 1994 (43%) (McLauchlin & Newton, 1995). Most common cause of outbreaks in UK in recent years has been due to consumption of prepared sandwiches served to patients at hospitals (Little *et al.*, 2008; Shetty *et al.*, 2009; Coetzee *et al.*, 2011; Little *et al.*, 2012).

### France

In France, the average incidence of listeriosis between 1948 and 1958 was 1.1 cases per year, whereas, between 1958 and 1970, it was 40.5 cases per year. Subsequently, the number of cases increased considerably between 1970 and 1975 to 165 cases annually. In Western France before 1975, the reported cases of listeriosis in humans did not exceed 15 cases per year, but there were 113 cases reported in 1975 and 54 cases in 1976. This increase was possibly due to the first outbreak observed in France. The increase was located in Anjou and involved 128 neonates and 39 adults. However, the source of infection was not determined. Most *L. monocytogenes* isolates belonged to serovar 4b (98%), whereas, serovar 1/2a was only 2%. Previously, serovar 4b was observed in high proportions, between 63% and 100% of *L. monocytogenes* clinical isolates, which indicates this serovar was predominant in France (Carbannelle *et al.*, 1978). In 1984, the incidence was 11.3 cases per million of population with 630 listeriosis cases being reported, of which 56% was associated with pregnancy and 44% with non-pregnancy cases. In 1986, human listeriosis cases increased to 807 (14.7 cases/million) and in 1987 decreased to 661 cases (11.9 cases/million). The proportion of pregnancy and non-pregnancy cases in these two years was similar. However, in 1988, the incidence decreased to 11 cases/million (Goulet *et al.*, 1990) and among 615 reported cases, the rate of pregnancy-associated cases was lower (43%) than non-pregnancy cases (57%). In 1987 in France, 384 *L. monocytogenes* isolates were characterised from 366 human cases. Serovar 4b was dominant (63%), followed by serovar 1/2a (22%) and 1/2b (13%); 8% of human serovar 4b isolates shared lysovar with food isolates. However, in 1988 among humans, serovar 4b isolates decreased to less than 50% (Espaze *et al.*, 1989).

A further, French outbreak occurred in 1989 and involved 14 individuals. However, the source of infection was not determined (Sutherland *et al.*, 2003). In the 1990s, six nationwide outbreaks were reported in France. The largest outbreak occurred in 1992 and involved 279 individuals, the fatality rate was 34% and most cases were associated with pregnancy (60%). The epidemic strain serovar 4b (pulsovar 2/1/3, related to the Swiss, Californian and Danish epidemic strain) was identified in 247 of 279 human isolates (89%), and 154 different food products (Goulet *et al.*, 1993; Jacquet *et al.*, 1995; de Valk *et al.*, 2000). According to French National Reference Center (NRC), the incidence of human listeriosis in France decreased from 6.3 in 1987 to 4.1 cases per million in 1997. However, two hospital laboratory surveys reported the incidence was 16.7 cases per million in 1987 and 14.9 cases per million in 1988 (Goulet *et al.*, 2001). In 1999, listeriosis surveillance was improved through new methods and the incidence of listeriosis in France decreased from 4.5 cases per million in 1999 to 3.5 cases per million in 2005, although in 2006, incidence increased to 4.7 cases per million, which was not due to any known outbreak (Goulet *et al.*, 2008).

### Germany

In Germany, 20 cases were described in 1945 and up to 1958, nearly a thousand cases had been diagnosed (Linell *et al.*, 1959). Between 1950 and 1956 in West Germany, 303 human cases of listeriosis were reported and in 1950 to 1975, 2000 cases were reported (Carbannelle *et al.*, 1978). At the beginning of 1960s in Germany, 80% of listeriosis cases were associated with pregnancy, however, from 1969 to 1985, the percentage decreased to 54% (McLauchlin, 1990b). In the early 1980s, the incidence of listeriosis cases per million habitants was highest in East Germany (3.36), France (3.27), Denmark (2.27), and Switzerland (2.05). The incidence was

lower in The Netherlands (1.69), Sweden (1.48), West Germany (0.96), USA (0.66), and Canada 0.62 (McLauchlin *et al.*, 1986).

In Germany, listeriosis has been a mandatory notifiable disease since 2001, and in this year, 217 cases were reported; an incidence of 2.6 cases per million population. In 2005, 510 cases were reported, with an incidence of 6.2 per million, but no outbreak was observed. Between 2001 and 2005, 1519 cases were reported and among all these cases, 15% were associated with pregnancy, with the most cases occurring in patients aged > 60 years (76%). Among 80 analysed isolates, 39 isolates shared serovar 1/2a, 38 shared serovar 4b and 3 shared serovar 1/2b (Koch & Stark, 2006). In 2009–2010, a multinational listeriosis outbreak occurred in Germany, Austria and the Czech Republic due to consumption of a red-smear cheese (Quargel) produced in Austria. The age of patients ranged between 57–89 years and most patients were male (76%). PFGE identified two different serovar 1/2a PFGE types in 14 and 20 human cases each (Fretz *et al.*, 2010; Rychli *et al.*, 2014). Between November 2012 and November 2015, 1765 human listeriosis cases were notified in Germany. The characterisation of 793 isolates identified a common source outbreak, where 66 patient isolates shared an identical PFGE pattern with *AscI* and *ApaI* enzymes, sharing serovar 1/2a and ST8 (Ruppitsch *et al.*, 2015).

#### *The Netherlands*

Between 1957 and 1976 in The Netherlands, 769 *L. monocytogenes* isolates were collected from human patients and clinically healthy individuals. The percentage of serovar 4b isolates (n=343) was 44.6%, whereas, the percentage of serogroup 1 (n=319) isolates was 41.8% (Kampelmacher & van Noorle Jansen, 1979). According to Kampelmacher (1962), human listeriosis in The Netherlands occurred among individuals who worked on farms or regularly had contact with animals. The annual incidence of listeriosis in 1981–1990 was 1.2 per million inhabitants, whereas, in 1991–1995, it was 0.7 per million. The decrease could be due to “effective microbiological monitoring of food sources, especially of soft cheeses” (Kampelmacher, 1962). In the period of 1976–1995, 207 isolates from listeria meningitis cases were collected; most of the patients were male (61%). Among 204 isolates, serovar 4b constituted 65% and 30% were 1/2a, 1/2b, or 1/2c (Aouaj *et al.*, 2002). Between 1995 and 2003, 283 patients were hospitalised with listeriosis, an incidence of 2.0 cases per million inhabitants annually: of all cases, 149 (53%) were men, 40% were adults between 65–79 years old, and 24 cases (8.5%) were associated with pregnancy. Among 159 clinical isolates from 1999–2003, serovar 4b was predominant (44%), followed by serovar 1/2a (36%) and 1/2b (16%). It was recommended that pregnant women should avoid soft cheeses, raw milk and smoked salmon (Doorduyn *et al.*, 2006).

#### *Switzerland*

A large listeriosis outbreak involving 122 individuals occurred in Switzerland between 1983 and 1987, due to raw milk cheese: the fatality rate was 28%, and 53% of the cases were associated with pregnancy (Bille & Glauser, 1988; Büla *et al.*, 1995). In a retrospective study (Boerlin *et al.*, 1996), 47 *L. monocytogenes* isolates sharing ET1 type serovar 4b isolated from humans, cheese and the processing plants from this Swiss outbreak were compared with isolates from 145 humans and 117 animals (collected between 1988 and 1993) and 8 animal isolates from 1986 that shared ET1 type serovar 4b. Among *L. monocytogenes* isolates collected from the post epidemic period, 28% belonged to humans and 37% to animals. In total, 144 isolates (45 human

isolates and 44 animal isolates post-epidemic and 47 human and 8 animal isolates from the epidemic period) belonging to ET1 type were further analysed by PFGE with *ApaI* and *SmaI*. Through combining both enzymes, 29 subtypes were obtained, where five were predominant (I–V). Among 144 isolates, PFGE subtypes II and IV were identified in human isolates, and subtypes I, II, IV and XX in cheese isolates were associated with the Swiss outbreak. In addition, the four subtypes encountered in these cheeses were identified among animal isolates and three of them were dominant. Moreover, subtypes I and II were the main subtypes identified among human (5%) and animal (8%) isolates collected in the post-epidemic period (Boerlin *et al.*, 1996). In Switzerland between 2011 and 2013, 93 *L. monocytogenes* isolates were collected from human individuals. Serovar 1/2a isolates were predominant (58 isolates), whereas, serovar 4b was less dominant (28 isolates), followed by serovar 1/2b (5 isolates) and 1/2c (2 isolates). PFGE yielded 70 different *AscI* types. The incidence over the last ten-year period fluctuated between 5.3 and 9.7 cases per million in Switzerland (Althaus *et al.*, 2014).

### *Spain*

In Valencia, between 1978 and 1988, 14 human cases of listeriosis were diagnosed, where *L. monocytogenes* serogroup 1 was predominant, whereas, in 1989, 9 cases were reported with serogroup 4 being predominant. This increase was possibly due to the first outbreak observed in Spain. Six isolates were characterised and shared serovar 4b; phage typing identified two types. Three of the isolates were identical to a listeriosis outbreak strain in ovines occurring in the province of Leon in 1990 (Vázquez-Boland *et al.*, 1991). On the island of Gran Canaria, only three cases of human listeriosis were reported between 1983 and 1987, whereas, five cases were reported during 1988–1991, and 19 cases in 1992. A common source outbreak was suspected due to this increase. Between December 1991 and May 1993 in Las Palmas, Gran Canaria, 24 cases of listeriosis were detected: 12 (50%) were pregnant-associated. Among 24 isolates, 21 (87.5%) shared serovar 4b and 2 (8.3%) serovar 1/2a. Of the 12 characterised isolates, seven had identical MEE and PFGE patterns. The majority of patients usually consumed fresh cheeses and RTE meat products (Elcuaz *et al.*, 1996). In a Spanish study, 51 human isolates recovered from different regions of Spain between 1989 and 1998 were characterised: serovar 4b was dominant with 38 isolates (74.5%), whereas, only 12 (23.5%) isolates shared serovar 1/2a. PFGE identified 29 *ApaI* pulsotypes, and two profiles were common with 11 and 8 isolates (Vela *et al.*, 2001). Subsequently, the incidence of listeriosis in Spain increased from 5.6 cases per million (2001 to 2007) to 9.3 cases per million in 2012, which is the second highest in the EU. An analysis of 17 human *L. monocytogenes* isolates, collected between 2006 and 2014, the majority from the region of Leon, Spain, found 58.8% of the isolates belonged to lineages I and III and 41.2% to lineage II. PFGE detected 14 *AscI* pulsotypes and MLST 11 STs. One ST, identified in 2012, was responsible for two outbreaks occurring in Northern Spain in 2013–2014 (Ariza-Miguel *et al.*, 2015).

### *Italy*

According to the Listeriosis Surveillance System in Italy, among human isolates in 2002 and 2003, serovar 1/2a was the most common (44%) followed by serovar 1/2b (32%) and serovar 4b (20%) (Gianfranceschi *et al.*, 2006). In a comparison of *L. monocytogenes* human isolates collected in 1990–1999 and 2002–2005, results revealed serovar 1/2a has increased, whereas, serovar 4b has decreased. In the second period (2002 to 2005), four different serovars were found among 57 human isolates: 1/2a (51%), 1/2b (16%), 1/2c (7%), and 4b (25%); and, PFGE

identified 23 *AscI* pulsotypes (Gianfranceschi *et al.*, 2009). Listeriosis in humans in Italy has been notifiable since 1993. In 2004 to 2006, the incidence of listeriosis was lower than in other European countries, 0.8 cases per million annually. However, during 2002 and 2003, an incidence of 1.3 cases per million was reported. In 2005 and 2006, the incidence was 1.4 per million in Lombardy and 4.5 per million in Tuscany (Mammina *et al.*, 2009b).

Between 1996 and 2007 in Lombardy and Tuscany regions of Italy, 54 human clinical isolates were collected. Overall, 25 (46.3%) *L. monocytogenes* human isolates shared serovar 1/2a, 23 (42.6%) isolates shared serovar 4b, and 6 (11.1%) isolates shared serovar 1/2b. PFGE identified 31 *AscI* pulsotypes, of which 19 pulsotypes were each represented by only one isolate (Mammina *et al.*, 2009a). Lombardy region accounted for 55% of listeriosis cases in Italy during 2006–2010, although, only 16% of the population lives in this region. The regional reference laboratory of Lombardy collected 134 *L. monocytogenes* isolates from 132 human cases in 2006–2010; 15 cases were associated with pregnancy and among 118 non-pregnant adults, 63 (53.4%) were females and 55 (46.6) males. From human isolates, 70 (52.2%) shared serovar 1/2a, 52 (38.8%) isolates shared serovar 4b, 9 (6.7%) isolates shared serovar 1/2b, and 3 (2.2%) shared serovar 1/2c. PFGE recognised 73 *AscI* pulsotypes, and MLST identified 22 isolates belonging to ST1 and ECI among serovar 4b. The high prevalence of listeriosis in Lombardy is possibly due to improved surveillance systems and a higher level of contamination in food products such as some soft-ripened cheeses (Mammina *et al.*, 2013).

#### Portugal

In Portugal, the incidence of listeriosis is underestimated, as the disease was not notifiable until 2014 and no active surveillance currently exists (Magalhães *et al.*, 2015). However, incidence increased from 1.4 cases per million inhabitants in 2003 to 2.3 in 2007. In an analysis of 95 *L. monocytogenes* human isolates collected between 1994 and 2007, 14.8% were associated with pregnancy. PCR group IVb was predominant and accounted for 71.6% of cases, whereas, group IIb accounted for 17.9% and group IIa 10.5%. The combination of *AscI* and *Apal* enzymes yielded 58 PFGE types including 18 pulsotypes, represented by 2 or more isolates. PCR group IVb yielded 14 pulsotypes, group IIb 3 pulsotypes and group IIa only one pulsotype. All PFGE pulsotypes were compared with the French human pulsotypes database and seven pulsotypes were indistinguishable from French pulsotypes: two pulsotypes were involved in the French pig tongue in aspic outbreak in 1992 (Almeida *et al.*, 2006 and 2010). In 2008–2012, 203 cases of listeriosis in Portugal were reported and the incidence ranged from 2.0 to 7.0 cases per million annually. Among all cases, 9.5% were associated with pregnancy and 46.4% of patients were over 65 years. Of the 203 isolates, the majority shared molecular serogroup IVb (77.8%), followed by serogroups IIb (14.7%) and IIa (7.4%); 91 PFGE types were identified with *AscI* and *Apal* enzymes. In the period 1994–2007, 26 of these types had already been isolated (Magalhães *et al.*, 2014). Between 2009 and 2012, an outbreak in Portugal involving 30 individuals occurred due to consumption of pasteurised milk cheese that harboured *L. monocytogenes* serogroup IVb. The fatality rate was 36.7%. Only two cases were associated with pregnancy and 13 (43%) cases were older than 65 years (Magalhães *et al.*, 2015).

### Denmark

In Scandinavia, the first human case of listeriosis was reported in 1929 in Denmark by Nyfeldt, who subsequently isolated *L. monocytogenes* from 13 humans up to 1940 (Bojsen-Møller, 1972). Between 1981 and 1987, *L. monocytogenes* was isolated from 138 individuals, and between 1958 and 1974, the annual incidence of listeriosis in Denmark was 2.3 per million, with 3.3 cases per million in 1981, and 1.5 cases in 1984. Subsequently, in 1985, the incidence increased to 4.3 cases, and increased again in 1986 to 8.4: in 1987, it was 5.3 cases per million. An outbreak occurred in 1985–1987 involving 35 individuals, where all 35 isolates shared serovar 4b and had identical phage type. Among this group, 15 cases were associated with pregnancy, 15 adults presented meningitis-encephalitis and the mortality rate was 20%. The mean age among outbreak patients was 47 years, whereas among non-outbreak patients mean age was 62 years. The source of infection was not identified (Samuelsson *et al.*, 1990; Nørrung & Skovgaard, 1993).

In a Danish study, 69 *L. monocytogenes* isolates were characterised from human cases and 30 isolates from meat products and slaughterhouse samples, collected in 1989–1990. Of these, 67% shared serogroup 4 and 33% belonged to serogroup 1. Among the 69 human isolates, one type was dominant, with 28 isolates belonging to serogroup 4, which were indistinguishable with three different typing methods (ribotyping, REA and MEE). The dominant type was not found among meat food and environmental isolates, but two types belonging to serogroup 4 and 1 from meat products isolates were identical to other human isolates with all three typing methods (Nørrung & Gerner-Smidt, 1993). Another study (Jensen *et al.*, 1994) identified a second Danish outbreak, which occurred in 1989–1990 and involved 26 patients, where the vehicle of transmission was probably blue mould cheese.

The incidence of listeriosis in Denmark increased markedly between 2002 and 2012 and became one of the highest incidences reported worldwide, from 5.0 cases per million inhabitants in 2002–2003 to 9.0 cases in 2012, with a peak of 18.0 cases in 2009. Between 2002 and 2012, 570 cases were reported, where 52% were females and 4% were associated with pregnancy. Among non-pregnant cases, the median age was 71 years and overall fatality rate was 26%. Most isolates, 334 (58%) belonged to lineage II and serovar 1/2a was dominant, whereas, 235 (42%) isolates belonged to lineage I, where serogroup 4 accounted for 193 (34%). The combined *AscI* and *ApaI* profiles yielded 178 pulsotypes, with 116 of them being identified in only one isolate each. One pulsotype was predominant with 82 isolates, followed by two pulsotypes with 40 isolates each. These pulsotypes, accounting for 122 cases, were closely related and multilocus sequence typing (MLST) revealed that both belonged to CC8. Furthermore, these two pulsotypes have been isolated in other countries *i.e.* Sweden, Finland and Norway (Kvistholm Jensen *et al.*, 2016).

### Norway

The first case of human listeriosis in Norway was reported in 1952. The fatal case was a 54-year-old farmer who became ill after he had cleaned the sheephouse. Two months later, a sheep died from listerial encephalitis (Bernander *et al.*, 1983). Before 1959, three human meningitis cases due to *L. monocytogenes* were reported (Bassøe *et al.*, 1959). In Norway, human listeriosis has been a notifiable disease since 1977 (Rosef *et al.*, 2012) and the first documented food-borne outbreak of invasive listeriosis occurred in Trøndelag during summer 1992, which involved six patients. Five samples of vacuum-packed RTE meat products from one manufacturer collected

from patients' refrigerators harboured *L. monocytogenes* and three samples taken from the processing plant were contaminated. The isolates from patients, meat products from the refrigerator, and the plant shared similar serovar and ET (Hellsnes *et al.*, 1992).

During the period 1977 and 2003, 289 cases of listeriosis were reported in Norway. The annual incidence of listeriosis ranged from 0.2 to 4.9 cases per million inhabitants during this period: male and female cases were represented equally and 39 (13%) cases were associated with pregnancy. The fatality rate was 45% in 1977–1987 and 41% in 1990–2000. Between 1977 and 2000, most listeriosis cases (70.7%) were from individuals aged 60 years or older. Within this same period, two outbreaks due to consumption of meat products harbouring *L. monocytogenes* occurred. The first in 1992, involved eight cases, and the second in 2000, involved four cases. In 2005, a third outbreak occurred at a hospital canteen, where three individuals ingested contaminated food (Antal *et al.*, 2007). In a retrospective study, 137 *L. monocytogenes* human isolates collected during 1992–2005 and 188 *L. monocytogenes* isolates collected during 1989–2002 from fish (n=90), poultry (n=26), meat (n=43), processing plants environments (n=29) were characterised by ribotyping. Most human isolates, 76 (55.6%) belonged to lineage II, whereas, 57 (41.6%) isolates belonged to lineage I. Among food and environmental isolates, lineage II was dominant. Of the 37 identified ribotypes, 22 ribotypes were found among human isolates and 33 ribotypes in remaining isolates. The two most common ribotypes were found among all sources and accounted for 58 (18%) and 54 (17%). The rate of listeriosis, during 1994 to 2003, fluctuated between 15 and 30 cases annually in Norway. However, in 2007, 50 cases were reported and this high rate was due to an outbreak involving 17 patients from a hospital who consumed soft cheese contaminated with *L. monocytogenes* (Rosef *et al.*, 2012).

#### *Finland*

In Finland, human listeriosis has been a notifiable disease since 1994, although, *L. monocytogenes* isolates have been submitted to the Finnish National Health Institute since the 1980s. Between 1990 and 2001, 20 to 50 human listeriosis cases were reported annually in Finland. During this period, 314 *L. monocytogenes* isolates (25 isolates linked with a butter-borne outbreak: Lyytikäinen *et al.*, 2001; Maijala *et al.*, 2001) were collected and most isolates were from male patients (57%), with 10.2% from pregnant-associated cases. Serovar 1/2a was predominant (53%), then, serovars 4b (27%), 3a (11%), 1/2c (6%) and 1/2b (3%). In 1990, serovar 4b was more dominant than serovar 1/2a (61%), but in 2000–2001, serovar 1/2a exceeded 60%. Among all isolates, 81 *AscI* PFGE types, including 11 PFGE types represented by five or more isolates, were detected. One PFGE type serovar 1/2a was predominant with 37 isolates (45.7%). This type was involved in an outbreak due to vacuum-packed cold-smoked rainbow trout in 1997 (Miettinen *et al.*, 1999b). One PFGE type serovar 1/2c (5%) was only found among male *L. monocytogenes* isolates (Lukinmaa *et al.*, 2003). In 2010, an outbreak of listeriosis caused by a specific *AscI* type, previously isolated in fish products, was investigated and in this year, 71 cases of human listeriosis were reported: the incidence was 13 cases/million, a pregnancy association of 7% and a fatality rate of 23%. Of 69 available *L. monocytogenes* isolates, serovar 1/2a was predominant (72%), with (23%) being serovar 4b. *AscI* PFGE type yielded 35 types and 13 (19%) isolates shared one PFGE type (*Lm96*). This type, identified in several European countries during 2010 and previous years, is commonly isolated from human and foodstuffs (Nakari *et al.*, 2014).



## 2.8.2 Human listeriosis in Sweden

In Sweden, veterinarians had primary knowledge about listeriosis and reported the disease in a large number of species (Lilleengen, 1942; Wramby, 1944; Nilsson & Karlsson, 1959; Andersson, 1961; Nilsson & Söderlind, 1974; Tham *et al.*, 1989). It was not until the 1950s that cases of listeriosis in humans were diagnosed in Sweden, especially among pregnant women and newborns (Ekelund *et al.*, 1962). In May 1958, Linell *et al.* presented what appears to be the first confirmed human listeriosis case, a 7-day-old child with *granulomatosis infantiseptica* and meningitis, at a meeting of the Pathology Association in Malmö (Linell *et al.*, 1959). A second report of human listeriosis was a fatal case of meningitis in an adult man (Brandt & Berg, 1959). Thereafter, during the 1960s, several cases of listeriosis in humans were diagnosed in different counties in Sweden; mainly perinatal listeriosis cases (Anonymous, 1960; Bergman *et al.*, 1960; Larsson, 1960; Ekelund *et al.*, 1962; Brorson *et al.*, 1968; Söderling, 1974; Larsson *et al.*, 1978a; Larsson *et al.*, 1978c; Niklasson *et al.*, 1978; Larsson *et al.*, 1979a). Since 1960, human listeriosis has been a notifiable disease in Sweden, and both clinicians and microbiological laboratories are required to report listeriosis cases to the County Medical Office and to the Public Health Agency, according to the Swedish Communicable Disease Act. Until 1999, the reporting system from clinicians and laboratories were analysed separately. As laboratory reports were voluntary and contained poor information from patients, only reports from clinicians were included in zoonosis reports (Romanus, 1984; SVA, 2000 and 2009). The Swedish Zoonosis Council, which was created in 1997 to oversee the zoonosis situation, was represented by the National Board of Health and Welfare, Swedish Institute for Infectious Disease control, National Food Administration, Swedish Board of Agriculture, National Veterinary Institute, and the Swedish Association of Local Authorities (SVA, 1999).

Between 1958 and 1974, 110 cases of human listeriosis were reported in Sweden: 46 cases (42%) were associated with pregnancy, and in two cases involving twins, and resulted in eight abortions and three stillborn. Among 37 neonatal cases born alive, 17 died (46%). Among 64 (58%) non-pregnant cases reported, 34 were male and 30 females. The fatality rate in this group was 31.3% (20 cases). The majority of cases occurred in adults aged between 40 and 70 years, with the highest fatality rate being among patients aged 50 to 70 years old with underlying diseases, such as leukaemia and alcoholism. The diagnosis was meningoencephalitis in 52 cases (16 fatalities) and septicaemia in 10 cases (4 fatalities). Incidence of human listeriosis was high in 1959 (16 cases) and 1960 (13 cases), but decreased each year between 1961 and 1971; the incidence was between 2 and 6 cases per year. In 1974, the incidence increased to 16 cases, and was similar to 1959. Among pregnancy-associated and non-pregnant cases, serogroup 1 and serovar 4b were equally distributed, but neither clinical diagnosis nor mortality was related to serogroups (Larsson *et al.*, 1978a; Larsson *et al.*, 1979a; Larsson *et al.*, 1979b). In 1975, two listeriosis outbreaks of nosocomial infection occurred in a hospital (two infants were infected in the first outbreak and five infants in the second), and was possibly due to a thermometer not being disinfected between patients (Larsson *et al.*, 1978b).

From 1975 to 1983, between 19 and 34 human cases were reported annually. Of 128 cases reported in 1979–1983, clinicians reported 62 cases and laboratories reported 66 cases. The highest listeriosis incidence was among patients over 60 years old (56%), with 9 (7%) cases being neonatal, and 38% of the cases had underlying diseases. During this period, the incidence

rate of listeriosis was three cases per million inhabitants (Romanus, 1984), with most cases being sporadic, although small outbreaks are reported (Larsson *et al.*, 1978a; Romanus, 1984).

In 1983, Bernander *et al.*, (1983) reported a probable outbreak of invasive listeriosis occurring in Västerås in the county of Västmanland during July to September 1981. Five cases were involved, two pregnant women and three males: the age of the patients varied between 28 and 73 years. Four of the patients lived in the same residential area and the fifth's parents lived in the area. An animal field with goats and sheep was located near the area, sometimes the animals got loose and moved within the residential area, and some families with children had rabbits that could borrow. *L. monocytogenes* serovar 4b was isolated from clinical samples and from samples of wool and straw. One sheep had a recent infection of mastitis and a milk sample harboured *L. monocytogenes*, serovar 4b. With the results from serotyping and epidemiological analysis, and that all cases occurred at the same time and same space, raised suspicion about a common route of infection.

During the 1990s, the reported number of cases of listeriosis was stable (between 25 to 35 cases annually): physicians reported 271 cases and the incidence was similar to the 1980s, with the highest incidence occurring among the elderly over 75 years (SVA, 1999).

The first documented sporadic case of foodborne listeriosis in Sweden (a woman suffering from meningitis) occurred in 1993 due to consumption of medwurst (Loncarevic *et al.*, 1997). *L. monocytogenes* was isolated from food products in the patient's refrigerator: vacuum-packed sliced pork brawn, sliced cooked medwurst, and berliner wurst of the same brand. At least three PFGE types were recovered, and one (in medwurst) was identical to *L. monocytogenes* PFGE type of the patient. This PFGE type was also isolated from the same products sold in the local retail store. The first documented outbreak of invasive listeriosis in Sweden occurred in the county of Värmland between 1994 and 1995 and involved nine patients. All patients had consumed gravad, cold- or hot-smoked rainbow trout/salmon vacuum-packed RTE products in the previous six months. Three different PFGE types all sharing serovar 4b were involved in the outbreak. The *AscI/ApaI* PFGE types were identified in both patients and samples from the same producer: gravad rainbow trout, cold-smoked rainbow trout and residues found in the packing machine. The fourth *AscI/ApaI* PFGE type was isolated from cold-smoked rainbow trout processed by another producer (Ericsson *et al.*, 1997).

In 1998, two sporadic cases of listeriosis were reported in Sundsvall, Västernorrland County. A hard cheese collected from one of the patient's refrigerator harboured < 100 cfu of *L. monocytogenes*, and Chinese cabbage samples from the second patient were heavily contaminated with *L. monocytogenes* (200–1000 cfu per gram). All isolates, both from patients and food, shared identical *AscI/ApaI* PFGE type and belonged to serovar 1/2a (Tham *et al.*, 1998).

Since 2000, the total number of reported cases, both from clinicians and laboratories were included and surveillance was based on passive case findings. However, an increase in reported cases was observed during the 2000s with a peak in 2001 (67 cases, and an incidence of 8.0 cases per million) and 2009 (73 cases). Between 1983 and 2012, the incidence of listeriosis increased by 2.5% annually, especially during the 2000s, and became one of the highest incidences

reported worldwide, from 4.0 cases per million inhabitants in 2002 to 9.6 cases in 2013. The number of pregnant-associated cases remained low, between 1–2 cases annually with a peak in 2001 (5 cases), 2007 (5 cases), 2010 (7 cases), and 2014 (5 cases). Among non-pregnant cases, the elderly (over 70 years old) were overrepresented. Between 2003 and 2015, the northern region had the highest incidence of listeriosis (*i.a.* Jämtland and Västernorrland counties), whereas, the three largest counties (Stockholm, Skåne and Västra Götaland) reported most of the cases. Fatality rate was high and stable, one-third of patients died within three months. As this group usually constitutes elderly with underlying diseases, the effect of listeriosis is complicated to estimate. Most cases were of domestic origin (SVA, 1999–2015).

In 2001, a large gastrointestinal outbreak of listeriosis in Hälsingland, Gävleborg County, linked to fresh cheese made of raw goat and cow milk on a summer farm caused febrile gastroenteritis in at least 120 people. Besides *L. monocytogenes*, some cheeses were contaminated with VTEC, ETEC and staphylococci. The isolation of *L. monocytogenes* was the most consistent finding from both patients and cheese samples, which were heavily contaminated with this pathogen, some cheese samples harboured over 10 million cfu per gram of *L. monocytogenes*. All isolates from clinical cases, cheeses, dairy animals, environment and equipment shared identical *AscI/ApaI* PFGE type and belonged to serovar 1/2a (Carrique-Mas *et al.*, 2003; Danielsson-Tham *et al.*, 2004). This is the first substantiated reported outbreak of listeriosis caused by raw milk cheese, where the epidemic strain was cultured from the milk of a dairy animal. The milk was used for fresh cheese manufactured on the farm. A goat with subclinical mastitis excreted 18,100 cfu *L. monocytogenes* per ml milk.

Since 2011, the typing routines of *L. monocytogenes* isolates from human cases have changed. The Public Health Agency of Sweden has used molecular serotyping and this new method has identified four molecular serogroups among all viable isolates. Serogroup IIa were most common (59% – 83%), followed by serogroup IVb (8% – 25%), serogroup IIc (2% – 16%) and serogroup IIb (3% – 6%) (SVA, 2011–2015; Personal communication: Hedenström, 2017).

Two invasive listeriosis outbreaks occurred between 2013 and 2015. In the first outbreak, 27 clinical cases were reported between May 2013 and April 2015 (7 cases 2013, 17 cases 2014 and 3 cases 2015) from nine counties, mainly in the Götaland and Svealand regions of Sweden. The majority of cases were reported from Skåne (10 cases) and Stockholm (6 cases) counties. The mean age of the 27 patients was 79 years (40 to > 90 years) and 67% were male. An epidemiological investigation revealed the source of infection as gravad and smoked fish products produced by one manufacturer. The clinical and food isolates were characterised by molecular serotyping, PFGE with two restriction enzymes *AscI* and *ApaI* and MLST. The causative strain in this outbreak belonged to serovar 1/2a, *AscI* PFGE type 0194 (EFSA nomenclature) and ST19. The second outbreak occurred from October 2013 to October 2014, and 16 counties were involved. The majority of cases were from Skåne (16 cases) and Västra Götaland (9 cases). During this period, 51 patients were affected (61% were female), including four maternal cases: there were seven fatalities. The consumption of contaminated cold cuts was suspected to be the source of infection and this outbreak was dominated exclusively by *AscI* PFGE type 0039 belonging to serovar 1/2a and ST7. The National Food Agency of Sweden analysed 70 meat product samples from three different manufacturers and found different PFGE types: five samples from the same manufacturer harboured the same *AscI* PFGE type 0039 as the

clinical samples (Public Health Agency of Sweden reports from 2015 and 2016; SLV report, 2016; Dahl *et al.*, 2017; Personal communication: Hedenström, 2017).

Since 2007, the incidence of human invasive listeriosis in Sweden has varied between 5.9 (2011) and 12.8 (2014) cases per million inhabitants: in 2015, there were 9.0 cases per million (Public Health Agency of Sweden, 2015). Although, contaminated food products are the main source of human listeriosis in Sweden, no official coordinated system exists for the surveillance of *L. monocytogenes* in food. Sampling is performed by national and local authorities, mostly at production units and retail levels. Sampling performed by the industry is not usually reported to the authorities (SVA, 2016).

As human listeriosis has increased in Sweden, five authorities jointly developed a five-year strategy in 2013 and 2014 for working with *L. monocytogenes*. These governmental authorities included the National Board of Health and Welfare, National Food Administration, Public Health Agency of Sweden, National Veterinary Institute, and the Swedish Board of Agriculture. The strategy document outlines certain measures these authorities consider particularly important (National Board of Health and Welfare, 2013). The *Listeria* research group at Campus Grythyttan, Örebro University, who reviewed the initial version of the strategy document, consider the measures listed in the strategy document are important. However, they recommend long-term basic research aimed at increasing understanding of *L. monocytogenes* and listeriosis should be included, and that characterisation of *L. monocytogenes* isolates from cases of human listeriosis should be obligatory.

In 2014, *L. monocytogenes* was included in the microbiological surveillance programme at the Public Health Agency of Sweden and included the typing of all *L. monocytogenes* isolates from human cases due to the last two outbreaks and the high incidence of listeriosis. In 2015, the Public Health Agency implemented a new molecular method for characterisation of clinical *L. monocytogenes* isolates: whole genome sequencing (WGS) (SVA, 2014 and 2015).

## 2.9 Contaminated food as source of listeriosis

Since first being identified, the disease listeriosis has been considered an occupational infection among veterinarians, farmers and butchers, or related to regular contact with animals (Sepp & Roy, 1963; Kampelmacher & van Noorle Jansen, 1979; Tham *et al.*, 1989). This disease usually affected single cases as the person was infected while assisting bovine or ovine partus or during slaughter. The possible relationship between human listeriosis and animal products was discussed during the Giessen symposium in West Germany on listeriosis in 1957, although, this was based on a description of a few cases with suspected source of infection. In the second symposium on listeric infection in 1962 in Montana, USA, researchers continued to deliberate about potential sources of human listerial infection (*e.g.* raw milk, egg and egg products, meat and meat products). Researchers also realised hygiene in kitchens and food-producing plants could pose a problem, and how food products with *Listeria* contaminates hands, utensils and kitchen tables. Concerns were also expressed that fish, in particular trout, could be fed raw minced meat contaminated with *Listeria* and that humans could be infected by consuming this fish (Kampelmacher, 1962). During a possible outbreak of listeriosis in Uppsala, Sweden, in the

beginning of 1960s, bacteriological investigations were undertaken on *e.g.* eggs and poultry but no *Listeria* were detected (Ekelund *et al.*, 1962); this investigation was two decades before the food-borne route for *L. monocytogenes* was confirmed.

## 2.9.1 Outbreaks of listeriosis and presence of *L. monocytogenes* in food

One of the first suspected human listeriosis outbreaks reported was in Halle, East Germany, between 1949 and 1957. The suspected sources of infection were raw milk, sour milk, cream and cottage cheese; approximately 100 individuals were implicated and the human *L. monocytogenes* isolates belonged to serogroup 1/2. Moreover, in 1954, an outbreak involving 26 individuals occurred in Jena, Germany, and one year later, an outbreak in 41 neonatals was reported from Prague, Czechoslovakia. The human *L. monocytogenes* isolates shared serogroup 1/2. In both outbreaks, the vehicle of transmission was not identified. In 1956, a correlation between the consumption of pork meat on a Soviet kolchoz and 19 human cases of listeriosis was suspected. In Bremen, Germany, two outbreaks occurred: in 1960–1961 (involving 81 individuals) and in 1963 (involving 20 individuals). In 1966, an outbreak of neonatal listeriosis occurred in Halle, East Germany, where 279 were affected and raw milk was suspected to be the source of infection; the human *L. monocytogenes* isolates belonged to serovar 1/2a. During the 1970s, three outbreaks were reported in the USA: the first in 1975 involving 6 neonatals, the second in 1976 among 20 non-pregnant individuals possibly due to raw salad; and, the third in 1979, also involving 20 individuals, where raw vegetables and cheese were suspected to be the sources of infection. The human *L. monocytogenes* isolates in the three American outbreaks belonged to serovar 4b (Kampelmacher, 1962; Ho *et al.*, 1986; McLauchlin *et al.*, 1986; Sutherland *et al.*, 2003).

Nevertheless, awareness that contaminated food could be the vehicle of infection by *L. monocytogenes* in humans was raised when Schlech *et al.* (1983) reported an outbreak occurring in Canada in 1981 that was caused by coleslaw salad. Seven non-pregnant individuals and 34 neonatals were implicated; the isolates belonged to serovar 1/2b (Schlech *et al.*, 1983). Thereafter, in the USA a further two outbreaks were reported, in 1983 in Massachusetts (49 cases), due to pasteurised milk, and 1985 in California (142 cases), due to soft cheeses; all isolates belonged to serovar 4b (Fleming *et al.*, 1985; Linnan *et al.*, 1988). Subsequently, several outbreaks due to contaminated foodstuffs have been reported in many countries. A wide variety of foods especially ready-to-eat foods with long shelf life have been identified as the vehicle for transmission of *Listeria*.

Since the 1970s, the Public Health Service of the UK has investigated the presence of *L. monocytogenes* in food and the environment. From 60,000 food items, 4% of RTE foods and 19% of food requiring cooking or reheating harboured *L. monocytogenes* (McLauchlin, 1996b). In the UK, an outbreak occurred between 1987 and 1989, probably due to imported pâté. Two serovar 4b subtypes were identified in humans and these subtypes corresponded to 96% of all isolates collected from a single manufacturer. However, a smaller amount of both subtypes had been isolated before and after the outbreak, and from other foodstuffs and manufacturers. In 1989, the English government advised vulnerable people against eating soft cheeses and pâté (McLauchlin *et al.*, 1991). In 1991, pregnant women were again advised to avoid infection from

food (McLauchlin, 1996b). A survey of food products between 1991 and 1992 revealed a significant decrease in *L. monocytogenes* contaminated soft cheeses and paté (one sample of 251 soft cheeses and 1 of 40 paté samples). However, other raw or minced meat products such as poultry samples (21 of 32), beef samples (9 of 26), lamb samples (8 of 20), pork samples (9 of 32), sausages samples (8 of 23) and raw fish samples (3 of 24) were more frequently contaminated with *L. monocytogenes* (MacGowan *et al.*, 1994). Most outbreaks in recent years have been due to contaminated sandwiches served in hospitals to vulnerable groups (Little *et al.*, 2008; Shetty *et al.*, 2009; Coetzee *et al.*, 2011; Little *et al.*, 2012).

In the French outbreak in 1992, the vehicle of infection was RTE products: 11,343 *L. monocytogenes* food isolates were collected and 220 food products harboured the endemic phage type. The epidemic strain serovar 4b, pulsovar 2/1/3 was detected in 154 different food products: pork tongue in aspic (n=112), other meat products (n=19), cheeses (n=12), diverse foods (n=11); and, even from food-processing environments (n=17) and animals (n=4) (Goulet *et al.*, 1993; Jacquet *et al.*, 1995). In 1992, a control measure previously only related to cheese production (section 2.9.2) was applied to all meat-processing manufacturers and expanded to all foodstuffs the year after (Goulet *et al.*, 2001). In 1993, contaminated rillettes harbouring *L. monocytogenes* serovar 4b caused an outbreak in France involving 39 individuals (Goulet *et al.*, 1998; de Valk *et al.*, 2000).

In France two large national samplings of RTE products (1993–1994, 5,809 samples: 1995–1996, 6,147 samples) analysed the presence of *L. monocytogenes*. In the first national study (1993–1994), *L. monocytogenes* was isolated from meat products (12.6% of 1533), seafood or fish products (10.4% of 841), prepared salads (5.2% of 1740), and dairy products (5.4% of 1695). The proportion of RTE foods contaminated with  $\geq 100$  cfu *L. monocytogenes* per g was 1.3% in 1993–1994 and 0.8% during 1995–1996. In the second national study in 1995–1996, the number of contaminated foods was slightly lower (Goulet *et al.*, 2001). During 1995–1996, PFGE type “a1” serovar 1/2a (187 isolates) was predominant among 287 *L. monocytogenes* isolates collected from five French pork slaughter houses combined with cutting plants. In total, 17 PFGE types with *Apal* enzyme were identified and two genotypes (with 186 and 16 isolates) were isolated from four of these plants. After sanitisation, some genotypes were still isolated from two plants (Giovannacci *et al.*, 1999).

During 2000 and 2001 in France, 398 *L. monocytogenes* clinical isolates and 271 food isolates were collected, but only 179 (45%) of the human isolates and 270 food isolates were characterised: seafood (79 isolates), dairy products (21), pork products (126) and other products (44). Among the food isolates, PCR serogroup IIa was predominant and mostly found in seafood isolates (89%), dairy products (57%) and pork products (39%). PCR serogroup IVb was also present in these products (5% in seafood, 14% in dairy products and 16% in pork products). In contrast, PCR serogroup IVb was predominant among human isolates (44%); IIa was less common (30%). Among human isolates, PFGE yielded 35 *AscI* PFGE types. Isolates from seafood (28 *AscI* PFGE types) and dairy products (9 *AscI* PFGE types) were the most diverse, whereas, isolates among pork products yielded 10 types, of which two types dominated that differed by one band below the size of 384.5 kb (Hong *et al.*, 2007).

In 2006, the occurrence of *L. monocytogenes* was reported in 13 French pork-meat processing plants (Thévenot *et al.*, 2006). Among 1028 isolates, four of the most common serovars that cause infection in humans were identified: serovar 1/2a (49.9%), serovar 1/2c (20.4%), serovar 1/2b (12%) and serovar 4b (8%). Furthermore, 83 *ApaI* PFGE types were identified and several were widespread in all 13 plants. The bacterium was probably introduced into the processing plants via contaminated raw meat (Thévenot *et al.*, 2005 and 2006). In France, the bacterium is mostly found in dairy products (frequently in soft cheeses), cooked pork meats (especially RTE dishes, such as potted meat and pork tongue in aspic), poultry, meats, smoked fish and seafood (de Valk *et al.*, 2001).

In Italy, two large national samplings of food products, environment, and human samples were taken in 1990–1999 and 2002–2005. Among *L. monocytogenes* food isolates serovar 1/2a was similar in both periods, whereas, among human isolates, serovar 4b decreased and serovar 1/2a increased. In the second period (2002–2005), serovar 1/2a was prevalent in humans (51%), food (39%) and environmental (51%) isolates, whereas, the prevalence of serovar 4b was 25% (humans), 10% (food) and 22% (environment). In the second period, serovar 1/2c was dominant in meat products (32%), especially Italian salami (69%), however, serovar 1/2a was frequently found in this product (23%). Around 80% of cheese isolates were from Gorgonzola cheese (blue-veined cheese) and among those isolates, serovar 1/2a was dominant (31%). Furthermore, in the second period, 85 *AscI* pulsotypes were identified among 558 food isolates. One of these pulsotypes was predominant in cheese, and was identical to 13 (22.8%) human isolates from the second period, comprising 57 human isolates (Gianfranceschi *et al.*, 2009). In 1997, a large outbreak of febrile gastroenteritis occurred in Italy and was associated with corn contaminated with *L. monocytogenes*. All reported cases (1566 students and staff from two primary schools) had eaten salad with corn and tuna at the school's cafeteria. *L. monocytogenes* serovar 4b with similar DNA profile were identified in 123 (among 141) fecal samples and one (among 40) blood sample (Aureli *et al.*, 2000).

In 2005–2008, the occurrence of *L. monocytogenes* was investigated in an Iberian pork-processing plant and slaughterhouse in Spain, which exported pork meat products to the USA. Isolates (n=541) were collected from raw and dry-cured products, carcasses, the environment, and equipment. Four of the most common serovars were found among the isolates: serovar 1/2a was dominant (68%), followed by serovar 1/2b (21%). Furthermore, in combined *AscI* and *ApaI* profiles, 29 pulsotypes were identified; 15 pulsotypes belonged to lineage II and 14 pulsotypes to lineage I. Among 349 (64%) raw products isolates, serovar 1/2a accounted for 74% of isolates and 25 pulsotypes were identified. However, only three pulsotypes were dominant (73%) and found in all types of samples (Ortiz *et al.*, 2010).

In Finland, 295 *L. monocytogenes* isolates were characterised from diverse food products from 41 producers in nine countries that were sampled between 1988 and 1999. Among the food isolates, 241 were from 28 Finnish producers and 54 food isolates were from Poland (19 isolates), Hungary (16 isolates), Estonia (7 isolates), France (5 isolates), Denmark (3 isolates), Norway (2 isolates), Belgium (1 isolate) and Spain (1 isolate): the isolates were from meat products (100), poultry (40), dairy (14), fish (76), vegetables (39), and other products (26). Six serovars sharing lineage I (1/2b, 4b), lineage II (1/2a, 1/2c, 3a) and lineage III (4c) were detected. *AscI* pulsotype yielded 61 types and *ApaI* pulsotype yielded 47 types, and a

combination of profiles of both enzymes yielded 66 pulsotypes. Of these, 10 pulsotypes were found in various food products. Furthermore, 17 pulsotypes were found in food products from two or more producers from several countries and isolated in different years *e.g.* in fish products (Autio *et al.*, 2002).

Between 1990 and 1997, the presence of *L. monocytogenes* at an ice cream plant in Finland was investigated: 1225 samples were collected from unpasteurised raw material and ice cream and 1320 samples from the processing environment and equipment. *L. monocytogenes* was isolated from 71 samples (2.8%). Two different *L. monocytogenes* serovars were identified among 41 isolates, serovar 1/2b was predominant with 37 isolates (90.2%) and serovar 4b had four isolates (9.8%). All serovar 4b isolates were from environmental/equipment samples and shared one PFGE type identified by three restriction enzymes; whereas, serovar 1/2b isolates were found at all sites, except raw materials and distributed into 11 PFGE types. Serovar 1/2b PFGE types mostly differed by one or two fragments and one predominant PFGE type accounted for 26 isolates (63%). Only two serovar 1/2b PFGE types were found in ice cream (Miettinen *et al.*, 1999a).

*L. monocytogenes* isolates from food products (n= 486) collected between 1997 and 1999 originated from 17 Finnish producers: five fish plants (206 isolates), eight meat plants (139 isolates), three dairy plants (38 isolates) and one poultry plant (103 isolates). Only 121 isolates were characterised: the most common serogroup was 1/2 (90%), with 4b only constituting 3.3%. PFGE with *AscI* yielded 42 types, PFGE with *SmaI* yielded 24 types and a combination with two enzymes yielded 46 types. Of these, 24 types were each represented by only one isolate. One PFGE type was isolated from three different plants: fish, meat and poultry but *L. monocytogenes* serovar 4b was only found in two plants (Aarnisalo *et al.*, 2003).

In Denmark in 1988, the prevalence of *L. monocytogenes* was 26% in minced beef and 47% in raw poultry (Skovgaard, 1988). In another Danish study (Skovgaard & Morgen, 1988) 67% of minced beef samples harboured *Listeria spp.* and 28% *L. monocytogenes*. From neck-skin samples of 17 carcasses of poultry, 94% harboured *Listeria spp.* and 47% *L. monocytogenes*. Serogroups 1 and 4 were common among the samples (Skovgaard & Morgen, 1988). In 1989–1990, 17 isolates were recovered from various sausages and minced raw meat manufactured by five Danish producers. In addition, 13 isolates from meat and the environment of a slaughterhouse were characterised. In meat products, 89% shared serogroup 4 and 11% serogroup 1; all isolates from the slaughterhouse belonged to serogroup 1 (Nørrung & Gerner-Smidt, 1993). In 2000, the occurrence of *L. monocytogenes* in a Danish turkey processing plant and turkey products was reported. *L. monocytogenes* was presented in 53 of 204 samples from the processing line and 12 of 101 samples from turkey products. Two common *Apal* PFGE types were found in both the plant and the products and identified in 9 and 1 human cases among 41 human isolates collected during and after the study period (Ojeniyi *et al.*, 2000).

The first documented case of food-borne listeriosis occurred in Sweden in 1993, due to the consumption of medwurst (Loncarevic *et al.*, 1997). Another meat-borne listeriosis outbreak occurred from October 2013 to October 2014 and included 49 patients. The National Food Agency of Sweden analysed 70 meat product samples from three different manufacturers and



found different PFGE types. Five of these samples harboured identical *AscI* PFGE type encountered in clinical samples (Public Health Agency of Sweden reports from 2015 & 2016).

In the USA, a multi-state listeriosis outbreak, linked to the consumption of cantaloupe melon, occurred in 2012: 146 cases were involved and the fatality rate was 20.5%. PFGE typing with *AscI* and *ApaI* restriction enzymes identified two closely related types serovar 1/2a and two closely related types serovar 1/2b in both cantaloupes and patients (Laksanalamai *et al.*, 2012; Lomonaco *et al.*, 2013).

The European Union Reference Laboratory for *L. monocytogenes* (EURL for *Lm*) coordinates a European network of 35 National Reference Laboratories (NRLs) that are responsible for typing food isolates. The PFGE standard operating procedure (EURL SOP) has been used by EURL since 2007. To facilitate the exchange of PFGE profiles between laboratories, EURL recommends the NRLs to use EURL SOP, and for this reason, the database EURL *Lm* DB was created. At the same time, the European Centre for Disease Prevention and control (ECDC) created the European Surveillance System (TESSy) to exchange PFGE profiles and epidemiological information from clinical isolates (Felix *et al.*, 2013; Michelon *et al.*, 2015).

As *L. monocytogenes* is capable of persisting for long periods in processing plants producing a wide variety of RTE foods and is resistant to heat, cold, salt and acidity, the food-processing environment has been the main source of contamination in the final product. *Listeria* has a large impact on global food industries, because the economic losses incurred every year are extremely high (Camargo *et al.*, 2016).

## 2.9.2 Contamination in cheeses

Cheeses are very complicated microcosms. The raw product, *i.e.* the milk, is produced in a contaminated environment, not least with faecal impurities. Cheese is manufactured at the optimum environment for growth of several pathogenic bacteria. Furthermore, this dairy product constitutes an excellent culture medium for pathogenic as well as non-pathogenic microorganisms. This is especially true for soft cheeses with their high moisture contents and water activity which offer favourable conditions for bacterial survival and growth. The cheese may become contaminated as a result of unsanitary conditions caused by food-contact surfaces, dirty hands, airborne organisms or packaging material. Cheese, particularly if prepared from untreated milk, has been associated with a wide range of infections.

In 1974, a study (Sipka *et al.*, 1974) in Yugoslavia on the survival of *L. monocytogenes* in white brined cheese made of unpasteurised cow's milk led to the recommended use of only pasteurised milk in the manufacture of fresh and ripened cheeses. However, in 1987 the first documented sporadic case of listerial meningitis due to heavily contaminated French pasteurised soft country cheese was reported in Britain. The implicated *L. monocytogenes* strain, serovar 4b, was found in the package consumed by the patient and was indistinguishable from the patient's isolate with phage typing. A further six cheeses from the same manufacturer were analysed without finding *L. monocytogenes* (Bannister, 1987). A second reported sporadic case in 1988 was associated with the consumption of vacuum-packed fresh goat cheese (Anari) from an

English manufacturer (X). Forty cheese samples from this producer were obtained from retail outlets and the processing plant during 11 months and 57% of the samples were contaminated with *L. monocytogenes*. All 73 isolates collected belonged to serovar 4b and were indistinguishable by phagetyping from the patient's isolates. During 1987 and 1988 in Britain, 533 clinical isolates (one per case) were collected, of which 378 (71%) belonged to serovar 4b. From 277 isolates that were further analysed, seven were indistinguishable by sero- and phagetyping from isolates found in the English manufacturer X (McLauchlin *et al.*, 1990c). Routine surveillance in the UK revealed the contamination of soft cheeses with *L. monocytogenes* decreased from 10% in 1987 to 1% in 1995 and even the cfu of *L. monocytogenes* per g declined (McLauchlin, 1996b). In 1997–1998, seven human cases infected by identical strains found in cheeses produced in England were identified (McLauchlin *et al.*, 2004).

In 1986 in France, the first control measure was established for cheese-processing manufacturers that sold cheeses to the USA. In 1988, after the Swiss cheese outbreak in 1983–1987, French authorities expanded the control to cover all cheese-processing plants (Goulet *et al.*, 2001). During the 1990s, three outbreaks occurred in France due to consumption of soft cheeses contaminated with *L. monocytogenes* serovar 4b. The first documented outbreak was associated with soft raw milk cheese, Brie de Meaux, in 1995 and involved 36 patients. The French NRC isolated the epidemic strain from four samples of Brie de Meaux among 2500 food isolates (Goulet *et al.*, 1995; Vaillant *et al.*, 1998; de Valk *et al.*, 2000; de Buyser, *et al.*, 2001). The second outbreak was in 1997 due to consumption of the soft raw milk cheese Pont l'Évêque, Livarot that infected 14 people (Jacquet *et al.*, 1998; de Valk *et al.*, 2000; de Buyser, *et al.*, 2001). In a case-control study in France during 1997 (de Valk *et al.*, 1998), 49% of enrolled sporadic cases of listeriosis could be attributed to eating soft cheese and that "Soft cheese may account for a substantial proportion of sporadic listeriosis" (de Valk *et al.*, 1998). The third documented outbreak in 1999 reported three listeriosis cases due to the consumption of the soft raw milk cheese Epoisses (de Valk *et al.*, 2000).

French investigators suggested a substantial part of the decrease in *L. monocytogenes* in RTE French food, including cheese, during the 1990s was related to control measures implemented at the food production level. Milking hygiene improved and cows with *L. monocytogenes* mastitis were not used for milk production. In addition, several precautionary measures during raw milk cheese preparation were taken (de Valk *et al.*, 2000; de Buyser *et al.*, 2001; Sanaa *et al.*, 2004). As a consequence of fewer contaminated ready-to-eat products, the number of listeriosis cases in France decreased by 68% from 1987 to 1997: the number of listeriosis cases per million population and year in France decreased from 5.2 in 1995 to 3.8 in 1996 (Jacquet *et al.*, 1998). "Because of the importance in the rural economy of food made from raw material of animal origin, especially in France, a great deal of effort has been placed on hygiene during production, transformation and distribution of cheese" (Sanaa *et al.*, 2004).

In Switzerland during 1983–1987, a Swiss soft cheese, Vacherin Mont d'Or, caused 122 cases of listeriosis (Bille & Glauser, 1988; Büla *et al.*, 1995). More than 200 soft cheeses of different brands and types, both domestic and imported, were analysed for the presence of *L. monocytogenes*. Among the soft cheeses analysed, 8 to 10% harboured *L. monocytogenes*. However, the only cheese isolates that matched the two particular epidemic human strains of *L.*

*monocytogenes* serovar 4b with two different phagetypes, were found in Vacherin Mont d'Or soft cheese (Bille, 1988; Bille & Glauser, 1988). Vacherin Mont d'Or was also sold in Sweden during the same period, however, currently Vacherin Mont d'Or is made of pasteurised milk (Personal communication: Tham, 2017). In 1986 in Switzerland, 9 of 70 soft cheese samples (7 smear and 2 white mould cheeses) harboured *L. monocytogenes* and 7 of 9 isolates belonged to serovar 4b. Four of the nine cheeses were made from pasteurised and five from raw milk (Breer, 1986). In 2005, a small cheese outbreak involving 10 individuals was detected in Norwest, Switzerland. A local manufacturer produced the implicated soft cheese, known as tomme cheese: all cheese and butter samples that were analysed of this brand harboured *L. monocytogenes* serovar 1/2a. Of two PFGE types identified by *AscI* and *Apal* enzymes, one type was predominant among human and cheese isolates (Bille *et al.*, 2006).

Between 1992 and 1994, 32 French unpasteurised soft cheeses, 32 French pasteurised soft cheeses, 20 unpasteurised and 2 pasteurised German cheeses were purchased on the German market. The presence of *L. monocytogenes* was higher (47%) in French unpasteurised cheeses, but was lower (9% each) in both French pasteurised and German cheeses (Eppert *et al.*, 1995). In 1999, German researchers analysed 374 cheese samples produced in six European countries from raw milk and pasteurised milk. The results confirmed samples from four countries were contaminated with *L. monocytogenes* (6.4%), including France (5 of 150), Germany (11 of 120), Italy (4 of 23), and Austria (1 of 10); from Switzerland 1 of 22 samples contained *L. innocua*, and from Denmark, no *Listeria* species were isolated (0 of 4). The incidence of *L. monocytogenes* was highest (7.6%) in semi-soft cheeses, followed by soft cheeses (6.3%) and hard cheeses (4.4%). Furthermore, 8% of contaminated cheeses were manufactured from pasteurised milk and 4.8% from raw milk. The high incidence of listeriosis in Europe was associated with red smear cheeses. In 2000, German authorities recalled 80 tons of soft and semi-soft red smear cheeses due to the presence of *L. monocytogenes* (Rudolf and Scherer, 2001).

In 2006–2007, a large listeriosis outbreak (189 cases) occurred in Germany due to the consumption of acid curd soft cheese made from pasteurised milk and ripened with a red smear. The implicated cheeses were produced by one manufacturer but sold under distinct brand names. Eleven cases were associated with pregnancy, among the adults, 62% were male, and the overall fatality rate was 14%. The *L. monocytogenes* isolates shared an identical *AscI* PFGE type serovar 4b (Koch *et al.*, 2010). In Austria between 1997 and 2000, the occurrence of *L. monocytogenes* in 181 small-scale Austrian cheese-processing plants was investigated: 182 *L. monocytogenes* isolates were recovered from 50 manufacturers (mostly from smear cheese and cheese) and 14 plants were heavily contaminated. Among 177 isolates, 88 (49.7%) shared serovar 1/2b, 47 (26.6%) isolates shared serovar 1/2a, 39 (22%) serovar 4b and 3 (1.7%) serovar 1/2c. Twenty-six manufacturers were contaminated with serovar 1/2a, 18 with serovar 1/2b, and 21 with serovar 4b. PFGE typing with *AscI* and *SmaI* enzymes identified 76 pulsotypes among 182 isolates. One *AscI* pulsotype serovar 4b was identified in eight manufacturers. However, among 41 human isolates from 1997–2002, no pulsotype could be associated with cheese manufacturers (Wagner *et al.*, 2006).

In 2009–2010, a multinational outbreak occurred in Germany, Austria and the Czech Republic, where two different *L. monocytogenes* clones serovar 1/2a caused 34 cases of listeriosis. The source of infection was a red-smear cheese (Quargel) produced in Austria. These cheeses were

also distributed to Poland and Slovakia, where about 50 and 60 tons of cheese were recalled (Fretz *et al.*, 2010; Schoder *et al.*, 2012; Rychli *et al.*, 2014). In 2015, an Austrian study reported 108 cheeses purchased through the Internet from seven European countries, only 19 cheeses (17.6%) were properly labelled and conformed to European requirements, and *L. monocytogenes* was detected in two cheeses (1.9%). A semi-hard cheese from The Netherlands yielded  $9.5 \times 10^3$  cfu/g at the end of expiry date and a French soft cheese < 10 cfu/g. According to EFSA, *L. monocytogenes* prevalence among 3452 soft and semi-soft cheeses analysed in the European Union was 0.47% at the end of expiry date (Schoder *et al.*, 2015).

In a Belgian study, *L. monocytogenes* isolates recovered from cheeses and human cases during 1990–1992 were characterised. The esterase typing revealed that among cheese isolates esterase type I B serovar 1/2a was dominant (44.2%), followed by esterase types II B and C serovar 4b (10.6%), whereas, among human isolates esterase types II B and C serovar 4b were dominant (40%) followed by esterase type I B serovar 1/2a (4.2%) (Gilot & Andre, 1995). In 1997, a fatal case of listeriosis associated with the consumption of French Camembert cheese was reported in Belgium: the isolates from both patient and cheese belonged to serovar 1/2a (Gilot *et al.*, 1997).

The first reported case of listeriosis due to the consumption of cheese in Italy was in 2003. Indistinguishable *L. monocytogenes* PFGE pulsotype, serovar 1/2b isolates, were recovered from the patient's blood sample and a Gorgonzola cheese made of pasteurised milk (Italian blue-veined cheese) that the patient had in the refrigerator. Cheeses from the same brand purchased in food stores and sampled from the production plant harboured 100 to 1200 cfu/g of identical pulsotype. Moreover, three previous studies conducted in Italy and USA, revealed 5–9% of Italian blue-veined cheese samples were contaminated with *L. monocytogenes* (Gianfranceschi *et al.*, 2006). Between 2002 and 2005 in Italy, 94 *L. monocytogenes* cheese isolates were collected, mostly from Gorgonzola cheese (blue-veined cheese), and 25 isolates from cheese-processing plants. Among the cheese isolates, serovar 1/2a was dominant (67 isolates) followed by 1/2b (20 isolates), 1/2c (3 isolates) and 4b (2 isolates). Serovar 1/2a was also dominant in the environment (92%), whereas, serovar 4b was only found in 8% of samples. One pulsotype was predominant both in cheese (44) and environmental (17) isolates (Gianfranceschi *et al.*, 2009).

Between 2011 and 2012 in Italy, 2205 cooked meat products and 1292 soft and semi-soft cheese products were collected from 12 manufacturers. Among 901 meat samples and 894 cheese samples, 1.7% of meat samples and 1.9% of cheese samples harboured *L. monocytogenes*. Of 894 meat samples and 398 cheese samples analysed at the end of their shelf life, 2.1% of meat samples and 1% of cheese samples harboured *L. monocytogenes*. One of four meat product types (Spalla cotta) was most frequently contaminated (16%), and among eight types of cheese products, three harboured *L. monocytogenes*: Taleggio (14.5%), Gorgonzola (4.2%) and Brie (2.3%). Of all 85 isolates characterised, serovar 1/2a was predominant (84.7%), serovar 1/2b, 7% and serovar 4b and 1/2c, 3.5% each. PFGE with *AscI* and *Apal* yielded 38 pulsotypes, with four pulsotypes accounting for 41.2% of all 85 isolates; these were associated with meat products or soft and semi-soft cheese (Iannetti *et al.*, 2016).

In Spain during 1992–1993, six types of regional cheeses from Asturias manufactured with pasteurised cow, ewe and goat milk were collected. In total, 101 samples of short-ripened cheeses were collected from five farms (home-produced) and one factory (manufactured) and

analysed. Nine samples of four homemade cheese types harboured *L. monocytogenes*, with one cheese type having the highest incidence of positive samples (7 samples). Thirty *L. monocytogenes* isolates from positive samples were further characterised: 10 isolates shared serovar 1/2a, nine isolates shared serovar 4b and 1/2b each. PFGE with *Apal* and *SmaI* enzymes yielded two PFGE types of serovar 4b, three types of serovar 1/2a and two types of 1/2b (Margolles *et al.*, 1996; Margolles *et al.*, 1998a; Margolles *et al.*, 1998b). In Galicia (Northwest of Spain), the prevalence of *L. monocytogenes* in a dairy processing plant was studied during one year: 376 samples from *i.a.* the environment, raw milk, cheese, untreated water, and butter were collected. Eleven (3%) samples harboured *L. monocytogenes*. Although, a high incidence was observed in raw milk, floors from raw milk reception areas and untreated water, no positive sample was found among finished products such as cheese and butter (Franco-Abuín *et al.*, 1996).

A retrospective study detected a listeriosis outbreak, involving 30 individuals, in Portugal between 2009 and 2012 and due to the consumption of cheese (queijo fresco) from cow and goat pasteurised milk. The *L. monocytogenes* isolates recovered from cheeses and the environment of a Portuguese manufacturer shared the same PFGE type serogroup IVb as the human isolates (Magalhães *et al.*, 2015).

In Denmark between 1988 and 1992, 26 *L. monocytogenes* isolates were collected from cheeses (soft, feta, hard, white moulded and blue-veined), raw milk (2 isolates) and the environment (9 isolates) from four Danish dairies. MEE identified one predominant type among these isolates: ET 4 serogroup 1, followed by ET 2 serogroup 4 and ET 4 serogroup 4, which was the second most frequent among human isolates. ET 1 serogroup 4 was commonly identified in human isolates but was only found in one cheese isolate. In 1989–1990, ET 1 and ET 4 serogroup 4 caused most human listeriosis cases in Denmark (73%) and were involved in two outbreaks in Switzerland and Massachusetts, USA, in the 1980s (Nørrung & Skovgaard, 1993).

In a Swedish study, 333 cheese samples from twelve countries were collected between 1989 and 1993. Only cheeses from three countries harboured *L. monocytogenes*; 18 (10.3%) samples of French cheeses were contaminated, with sixteen samples sharing serogroup 1 and two serogroup 4, and one sample each from German and Italian cheeses shared serogroup 1. Of 30 French soft cheeses made of raw milk, 13 (43.3%) tested positive for *L. monocytogenes*. The majority of the positive French cheeses harboured < 100 cfu *L. monocytogenes* serogroup 1/2 per g; however, the serovar was 4b in two cheeses with the largest number (2300 and 100,000 cfu/g). The French cheeses positive for *L. monocytogenes*, Brie de Meaux, Pont l'Évêque, Livarot and Epoisses, were available on the Swedish market between 1989 and 1993 (Loncarevic *et al.*, 1995; Personal communication: Loncarevic, March 2017). During this time, Brie de Meaux was one of the most popular soft raw milk cheeses sold in Sweden (Personal communication: Tham, 2017).

During 1980s and 1990s, large outbreaks and sporadic cases of food-borne listeriosis due to closely related strains belonging to serovar 4b were reported (Kathariou, 2002). Between 1983 and 1987, a large human listeriosis outbreak involving 122 individuals occurred in Switzerland due to contaminated soft cheeses (Bille & Glauser 1988). In 1985, a large outbreak involving 142 individuals was reported in California, USA, and was due to soft cheese (Linnan *et al.*, 1988). A Danish outbreak involved 26 individuals and occurred during 1989–1990: the vehicle

of transmission was probably blue mould cheese (Jensen *et al.* 1994). In the French 1992 outbreak, the epidemic strain serovar 4b (pulsovar 2/1/3), which infected 247 individuals, was isolated from several RTE products, *i.a.* 12 cheeses. It also caused disease in previous years with 6 to 27 cases annually in France (Jacquet *et al.*, 1995). The epidemic cheese-borne strains from Switzerland, California, Denmark and France belong to the “long Swiss epidemic phagetype” (known from the Swiss soft cheese outbreak) and were closely related regarding serotyping, ribotyping, phagotyping, MLE and PFGE (Buchrieser *et al.*, 1993; Rocourt *et al.*, 1993; Tham, 1993; Jacquet *et al.*, 1995; Salvat *et al.*, 1995; Kathariou, 2002). This phagetype was common among the Swedish 4b human isolates (63.5%) during the period 1976–1985 (Ericsson *et al.*, 1996).

In Philadelphia, USA, a listerial outbreak in 1986–1987 was investigated: 36 individuals (32 non-pregnant adults and 4 pregnant cases) were involved and there were 16 fatalities (44%). *L. monocytogenes* was isolated from Brie cheese eaten by one patient that matched with patient’s isolate (Schwartz *et al.*, 1989). Between 1986 and 1988, a surveillance study in the USA investigated the prevalence of *Listeria* species in dairy environments and products. Of 1370 plants inspected, 37 (2.7%) had dairy products contaminated with *Listeria* and 29 plants were contaminated with *L. monocytogenes* (Kozak *et al.*, 1996).

Between 2001 and 2003 in New York State, USA, 495 *L. monocytogenes* isolates originating from human cases (120), food products (74), animal farms (221) and urban/natural environments (80) were collected. PFGE with *AscI* yielded 244 types and PFGE with *ApaI* yielded 266 types: a combination with two enzymes yielded 310 PFGE types. Of those, a majority (n=235, 47.5%) types were each represented by one isolate only. Two PFGE types serovar 4b were predominant, one of which was predominant among humans (16 isolates, 13.3%); however, this was only isolated from two different foods: 2 turkey breast samples and 2 white cheese samples. This type was responsible for a multi-state outbreak in the northeastern USA in 2002, where 46 individuals were affected due to consumption of sliced turkey meat. The second most common PFGE type was identical to the “long Swiss epidemic phagetype” and accounted for 15 isolates identified from all sources and during the entire study period. This strain caused multinational listeriosis cheese-borne outbreaks, *e.g.* in California (1985) and Switzerland (1983–1987) (Fugett *et al.*, 2007).

### 2.9.3 Contamination in fish

One of the most frequently *L. monocytogenes*-contaminated RTE food is vacuum-packed gravad and smoked fish (EFSA 2011; Kramarenko *et al.*, 2013, 2016). *L. monocytogenes* has been isolated from gravad, hot and cold-smoked rainbow trout (*Oncorhynchus mykiss*) and salmon (*Salmo salar*) around the world. Løvdal (2015) summarises results from scientific publications from 2000 and highlights a prevalence of *L. monocytogenes* in the retail sector for cold-smoked salmon of 0–61%, averaging 9.8%. The serovar most often encountered in these products is serovar 1/2a (Johansson *et al.*, 1999; Rørvik, 2000; Vitas *et al.*, 2004; Meloni *et al.*, 2009; Kramarenko *et al.*, 2013; Gyurova *et al.*, 2015; Kramarenko *et al.*, 2016).

During the 1990s, outbreaks of listeriosis occurred in Sweden (Ericsson *et al.*, 1997) and in

Finland (Miettinen *et al.*, 1999b) due to rainbow trout. This generated an increased global interest to determine the prevalence of *L. monocytogenes* in RTE fish products, processing plants and environments. For example in Belgium (Van Coillie *et al.*, 2004; Uyttendaele *et al.*, 2009), Brazil (Cruz *et al.*, 2008), Canada (Kovačević *et al.*, 2012), Estonia (Kramarenko *et al.*, 2013, 2016), Finland (Lyhs *et al.*, 1998; Johansson *et al.*, 1999), France (Besse *et al.*, 2008; Beaufort *et al.*, 2007), Greece (Soutos *et al.*, 2014), Iran (Fallah *et al.*, 2013), Ireland (Corcoran *et al.*, 2006; Dass *et al.*, 2011), Island (Gudmundsdottir *et al.*, 2005), Italy (Latorre *et al.*, 2007; Di Pinto *et al.*, 2010), Japan (Inoue, *et al.*, 2000; Nakamura *et al.*, 2004), Nigeria (Salihu *et al.*, 2008; Meloni *et al.*, 2009), Poland (Medrala *et al.*, 2003; Kwiątek, 2004), Spain (Dominguez *et al.*, 2001; Vitas *et al.*, 2004; Cabedo *et al.*, 2008; Garrido *et al.*, 2009; Gonzalez *et al.*, 2013), Norway (Rørvik *et al.*, 2000), Sweden (Peiris *et al.*, 2009; Thisted Lambertz *et al.*, 2012).

Gravad and cold-smoked salmons are vacuum-packed RTE products with a generous best before date of normally 4 to 5 weeks and constitute an optimal environment for the facultative anaerobic, psychrotrophic organism *Listeria*. The temperature of the cold-smoke, salt content and refrigeration storage will not prevent the growth of *L. monocytogenes* (Ben Embarek, 1994; Rørvik, 2000; Vaz-Velho *et al.*, 2001). The near neutral pH values (6.0–6.5) and the high  $a_w$  values (0.96–0.98) in smoked salmon create an environment suitable for *L. monocytogenes* (Rocourt *et al.*, 2000; Uyttendaele *et al.*, 2009). However, smoke, salt, and vacuum packaging extends shelf life and renders the food products attractive to consumers. Nevertheless, the occurrence of *L. monocytogenes* in vacuum-packed smoked fish products constitutes a serious food safety risk (Uyttendaele *et al.*, 2009; Tocmo *et al.*, 2014; Løvdal *et al.*, 2015).

The prevalence of *L. monocytogenes* in raw fish is generally low (Autio *et al.*, 1999; Johansson *et al.*, 1999; Fonnesbech Vogel *et al.*, 2001; Markkula *et al.*, 2005). However, the possibility that raw fish is a source of contamination in the processing equipment and environment cannot be excluded (Rørvik, 2000; Gudmundsdottir *et al.*, 2005). *L. monocytogenes* probably arrives at the plant via raw material and establishes itself in the processing equipment and processing environment (Huss *et al.*, 2000; Fonnesbech Vogel *et al.*, 2001; Tompkin 2002; Medrala *et al.*, 2003; Cruz *et al.*, 2008). Thus, *L. monocytogenes* will remain and contaminate the products in the final stages of fish processing during brining, slicing and packaging (Autio *et al.*, 1999; Fonnesbech Vogel *et al.*, 2001). Some strains of *L. monocytogenes* may be specially adapted to the processing plant environment, and are difficult to sanitise through standard hygiene procedures (Fonnesbech Vogel *et al.*, 2001). Especially salting and slicing machines are reported as difficult to clean and keep *Listeria*-free. Cold-salted and smoked products are usually sliced, causing contamination of the slicing machine (Johansson *et al.*, 1999). However, there are no differences in the adherence of persistent and non-persistent strains over extended cultivation times (Lundén *et al.*, 2003).

As RTE fish products are often contaminated by *L. monocytogenes*, outbreaks would be expected to be more frequent. One reason for the absence of fish-borne outbreaks could be that counts of *L. monocytogenes* frequently follow the food safety criterion of 100 cfu/g during the product's self-life. In addition, the long incubation period for listeriosis renders it difficult to determine the food source (Tompkin, 2002). Another theory presented by Rocourt *et al.* (2000) is that outbreaks due to fish consumption are rare because factories producing fish products are often smaller than factories processing milk and meat products.

Vacuum-packed gravad and cold-smoked salmon should be considered possible sources of listeriosis in Sweden. Since the mid 1990s, cold-smoked and gravad salmons have been more frequently contaminated with *L. monocytogenes* than cheeses and their consumption has increased in Sweden, and in several other countries (Loncarevic *et al.*, 1998a; Tompkin *et al.*, 2002; Knapp *et al.*, 2007; Thisted Lambertz *et al.*, 2012, 2013).

In 1980, an outbreak of listeriosis in New Zealand involved 19 perinatal cases and 3 adults. The *L. monocytogenes* isolates belonged to serovar 1/2a. Shellfish and raw fish were suspected as the sources of infection, as mothers frequently consumed these products, although no foods were analysed (Lennon *et al.*, 1984; Sutherland *et al.*, 2003). Another small outbreak in New Zealand, due to the consumption of smoked mussels produced in a local manufacturer, occurred in 1991–1992. The *L. monocytogenes* isolates from four human cases and mussels shared indistinguishable *Apal* and *SmaI* PFGE type serovar 1/2a. Analysis with MvLST indicated this type shared ST01-N, which is frequently identified in seafood, seafood-processing plants, and human cases in New Zealand (Brett *et al.*, 1998; Cruz *et al.*, 2014).

In Switzerland during 1988–1990, 990 imported fish products were collected and *L. monocytogenes* was isolated with high prevalence in gravad salmon (26.9%), cold-smoked fish (13.6%), and hot-smoked fish (8.9%). Serovar 1/2b was predominant (58.6%) in these products, 1/2a was 19.8% and 4b was 14.4% (Jemmi, 1990). In another study, 58 (8.4%) of 691 hot-smoked fish and 49 (11.3%) of 434 cold-smoked fish harboured *L. monocytogenes*. In hot-smoked fish samples, 50% of isolates shared serovar 1/2b, 39.6% shared serovar 1/2a and 1.7% shared serovar 4b. In cold-smoked fish samples, serovar 1/2b constituted 38.8% of isolates, 22.4% was serovar 1/2a and 26.5% was serovar 4b (Jemmi *et al.*, 1992).

In 1993–1995 in Switzerland, 72 *L. monocytogenes* isolates were collected from diverse fish products from six countries and 12 producers: Norway (1), Denmark (2), New Zealand (1), Great Britain (4), France (3) and Germany (1). Only 23 fish isolates were compared with 47 human isolates collected in 1993–1994. Serovar 1/2a was found in 47 (65%) fish isolates, and in 17 (36%) human isolates. In contrast, *L. monocytogenes* isolates serovar 4b accounted for 21 (45%) isolates in humans and one (1.4%) in fish: even serovar 1/2b was prevalent in humans (8 isolates) and in fish (2 isolates). Serovar 1/2c (4 isolates) and 3a (2 isolates) were only found among fish isolates. Serovar 7 was found in 12 fish isolates. Human and fish isolates shared three electrophoretic types (ETs) that were common among human isolates. One of these ETs is frequently associated with meat and meat products in Switzerland. Among fish isolates, PFGE identified 13 *Apal* types with only one type found in both fish and human isolates (Boerlin *et al.*, 1997). During 1992–2000 in Switzerland, imported and exported meat and fish products of various kinds and from different production plants were analysed for *L. monocytogenes*. The products contaminated with *L. monocytogenes* were marinated fish (38%), fermented sausages (15%), and cold-smoked fish (14%). The level of *L. monocytogenes* was < 100 cfu/g with one exception; one smoked salmon sample harboured 1100 cfu/g. The dominant serovar in meat and fish samples was 1/2a (57%); serovar 4b was present in 10% of samples (Jemmi *et al.*, 2002).

In a French study, 1010 samples of vacuum-packed sliced cold-smoked salmon, produced by nine French plants during 2001 to 2004 were collected: 104 (10.3%) samples harboured *L.*



*monocytogenes*. Altogether, 693 isolates were characterised and 72.4% shared serovar 1/2a, 16.7% shared serovar 1/2b, and 10.9% shared serovar 1/2c; no serovar 4b isolate was found (Beaufort *et al.*, 2007). The number of invasive listeriosis cases due to cold-smoked salmon consumption in France is predicted to be 307 per year. Even if this calculation is uncertain, it is based on scientific observations and can be used to manage the risk linked to the consumption of contaminated cold-smoked salmon (Pouillot *et al.*, 2009).

A national case-control study of sporadic listeriosis cases in England and Wales during 1990–1992 revealed listeriosis was associated with high-risk foods including shellfish and cooked chicken (McLauchlin, 1996b). Furthermore, the United Kingdom requested a national sampling survey for analysing the presence of *L. monocytogenes* and among 1344 cold-smoked fish, 236 (17.4%) products harboured *L. monocytogenes*, all samples had a level of < 100 cfu/g (ACMSF, 2009). In Ireland, 26 *L. monocytogenes* isolates recovered from vacuum-packed smoked salmon products were characterised in 2001. PFGE identified six *Apal* types and a single PFGE type serovar 1/2a accounted for 17 (65%) isolates. Only two isolates shared serovar 4b with identical PFGE type but different MLVA profiles (Corcoran *et al.*, 2006; Murphy *et al.*, 2007).

In Belgium, *L. monocytogenes* was detected in 27.8% (25/90) smoked fish samples (Uyttendaele *et al.*, 2009). In Austria, among 41 human listeriosis cases occurring in 1997–2000, 31% had consumed cold-smoked salmon, raw milk products and soft cheeses. Among 41 *L. monocytogenes* human isolates, 37 pulsotypes were identified. The human isolates and 83 food isolates collected from 1986 onwards were compared. One pulsotype serovar 1/2a from salmon was identical to one human isolate. A second pulsotype serovar 1/2a from salmon was identical to two human isolates and a third pulsotype serovar 4b from raw salmon was identical to one human isolate (Wagner & Allerberger, 2003).

In a Spanish study, 30 *L. monocytogenes* isolates were collected from smoked salmon products between 1995 and 2005. In total, 17 (56.7%) isolates belonged to serovar 4b and 13 (43.3%) to serovar 1/2a. From 33 Spanish clinical isolates, 10 isolates sharing pulsotype 1 serovar 4b were identical to seven salmon isolates, and one human isolate belonging to pulsotype 16 serovar 1/2a was identical to five salmon isolates (Garrido *et al.*, 2008).

Between 2002 and 2005, 72 *L. monocytogenes* fish isolates were analysed in Italy. From 17 raw fish isolates, 11 (65%) shared serovar 1/2a, 3 (18%) shared serovar 1/2b and 1 (6%) shared 4b. Among 55 RTE fish isolates, with 80% from smoked salmon, 34 (62%) shared serovar 1/2a, 6 (11%) serovar 1/2b and 10 (18%) 4b. Two *AscI* pulsotypes were predominant in RTE fish isolates and one was identical to a human isolate (Gianfranceschi *et al.*, 2009).

In Japan, 95 RTE seafood products were examined for *L. monocytogenes* between 1999 and 2000. *L. monocytogenes* was isolated from 12 (13%) of cold-smoked salmon or trout products. The amount of *L. monocytogenes* in all products was < 100 cfu/g. The predominant serovar was 1/2a (Nakamura *et al.*, 2004). In 2004–2008, 14 (12%) of 116 minced tuna samples, 15 (9%) of 164 cod roe samples, 7 (5.7%) of 123 salmon roe samples and 1 (3%) of 33 smoked salmon harboured the pathogen. Serovar 1/2a was predominant, followed by serovars 1/2b and 4b (Miya *et al.*, 2010).

In Brazil, a gravad salmon processing plant that used raw fish from Chilean ocean farm-raised salmon was studied during 1999–2000. Of 415 samples, 48% of raw salmon samples and 36% of vacuum-packed samples were contaminated: 179 *L. monocytogenes* isolates were characterised. Most isolates shared serogroup 1 (73%) and serogroup 4 was mainly found in the final product. Restriction enzyme *AscI* generated 28 PFGE types, *ApaI* generated 30 PFGE types, and a combination of both enzymes yielded 61 types (Cruz *et al.*, 2008).

In the Scandinavian countries, RTE vacuum-packed fish products are popular foods (Johansson *et al.*, 1999; Noriega Orozco, 2000; Lukinmaa *et al.*, 2003; Kvistholm Jensen *et al.*, 2016). Between 1996 and 1998 in Finland, 110 samples of diverse vacuum-packed fish products from 12 producers were collected. Twenty-two samples from six producers harboured *L. monocytogenes*, either serovar 1/2a (19 isolates) or 4b (3 isolates). Five of 30 cold-smoked samples (17%), 16 of 32 cold-salted rainbow trout (50%), and one from 48 hot-smoked fish samples (2%) were positive for *L. monocytogenes*. As most samples from one producer contained > 100 cfu/g of *L. monocytogenes*, a further 200 samples were collected from the plant and products. Simultaneously, 55 samples of raw fish were collected from six farms supplying fish: no *L. monocytogenes* was isolated from raw fish samples. One dominant PFGE type was isolated from samples of four producers, and this type has been identified among human isolates in Finland (Johansson *et al.*, 1999). In 1998, a febrile gastroenteritis outbreak (5 cases) occurred in Finland due to contaminated vacuum-packed cold-smoked rainbow trout containing  $1.9 \times 10^5$  cfu/g of *L. monocytogenes*. Isolates from the implicated food and stool swabs belonged to serovar 1/2a and shared identical *AscI* and *SmaI* PFGE types (Miettinen *et al.*, 1999b).

In 2001, the presence of *L. monocytogenes* in two freshwater fish farms in Finland was investigated and several samples were collected from earth canal, earth ponds, a concrete-plastic tank, slaughterhouses, and slaughtered fish. Serovar 1/2a was shared by all *L. monocytogenes* isolates from both farms. Five *AscI* pulsotypes were identified, four were isolated from farm A and the fifth from farm B. Similar pulsotypes were found in both primary food production facilities and in final fish products. Three pulsotypes from farm A had been previously isolated from fish products (Katzav *et al.*, 2006).

During an outbreak of listeriosis in 2010 in Finland, 240 food samples, including 186 (78%) fish samples, were analysed and from 323 *L. monocytogenes* isolates, 162 (50%) were isolated from two fishery processing plants. The same year, an official survey collected 257 samples of various foodstuffs (*i.e.* gravad and cold-smoked fish samples) but only fish samples were positive for the presence of *L. monocytogenes*, 20 (16%) of 126 fish samples. Between these two studies, 307 *L. monocytogenes* isolates from 204 food samples collected in both studies were characterised, of which 162 (79%) were fish samples. The serovar 1/2a PFGE type (*Lm96*) that caused the outbreak was identified in 76 (37%) of the food samples and isolated from three fish processing plants, one of which was located in Finland (Nakari *et al.*, 2014).

In Denmark, cold-smoked fishery products are considered an important source of listeriosis in humans (Kvistholm Jensen *et al.*, 2016). During 2013–2015, two outbreaks involving 20 cases, including eight fatalities (40%), occurred in Denmark and were associated with smoked fish consumption. All cases belonged to known risk groups. The two epidemic strains involved were serotype IIa, ST391 and serotype IVb, ST6 (Gillesberg Lassen, 2016).

In Norway, in 1991–1992, the frequency of *L. monocytogenes* in vacuum-packed smoked salmon was 11%, and in raw fish was 17% at a salmon processing plant, slaughterhouse and smokehouse. MEE identified 11 ETs and one was predominant (ET-6). This ET was the most common type in Norway and caused a small outbreak due to consumption of vacuum-packed meat sausage in 1992 (Kolstad *et al.*, 1992; Rørvik *et al.*, 1995). Between 1992 and 1997, 261 isolates were collected from seafoods, seafood-processing plants and seawater (from 55 different producers) and compared with 44 human isolates collected in 1992–1996. Nine ET were detected in all sources and four ET belonging to serogroups 4 and 1 (2 ET each) were most commonly represented (Rørvik *et al.*, 2000). In another Norwegian study, 65 *L. monocytogenes* isolates recovered from three fish-processing plants were characterised with MLVA. Of 15 MLVA profiles identified, nine, including profile 07-07-09-10-06, corresponded to MLVA profiles from Norwegian human isolates. MLVA profile 07-07-09-10-06 is regularly found in Norwegian (30%) and Swedish (4.3%) patients (Lindstedt *et al.*, 2008).

In Iceland, 38 samples of vacuum-packed gravad salmon, cold-smoked salmon and trout from 10 different local companies were analysed. Five (13%) of all samples and four (28.6%) of 14 gravad salmon samples harboured *L. monocytogenes* (Noriega Orozco, 2000).

A case of gastrointestinal listeriosis occurred in 1993 in Sweden due to the consumption of gravad salmon. The PFGE type, with *ApaI* and *SmaI* enzymes, of *L. monocytogenes* faecal isolates were identical to the PFGE type of the *L. monocytogenes* isolates identified in salmon kept in the patient's refrigerator. This PFGE type serovar 4b was previously identified in a Swedish salmon plant and was identical to the French clone that caused an outbreak due to consumption of pig tongue in aspic in 1992–1993 (unpublished data). In 1993–1994, the National Food Administration of Sweden analysed the occurrence of *L. monocytogenes* in RTE fishery vacuum-packed products. The products were from 15 manufacturers and purchased in Stockholm. Four (4%) samples out of 100 harboured *L. monocytogenes*: one gravad rainbow trout and three hot-smoked mackerel. Furthermore, 50 samples of raw fish from three countries were analysed: Sweden (31), Norway (17) and Denmark (2). One fish sample from Sweden and one from Norway were positive and both shared *L. monocytogenes* serogroup 4 (Westöö, 1994). During August and December 1994, 64 vacuum-packed fish samples from 15 different brands were collected in southern Sweden. Fifteen (23%) samples harboured *L. monocytogenes* (gravad, hot/ cold-smoked rainbow trout and hot-smoked mackerel): one hot-smoked mackerel sample contained 11,100 cfu/g (Detmer & Blomgren, 1995).

During 1993 and 1994, 150 samples of vacuum-packed fish purchased in Sweden were collected and 190 *L. monocytogenes* isolates from 16 positive samples were isolated. Five samples harboured isolates belonging to serogroup 4, nine samples were serogroup 1/2, and two samples serogroup 3, but one also harboured serogroup 1/2. Twelve (20.7%) of 58 gravad, 3 (11.5%) of 26 cold-smoked and one of 66 hot-smoked samples harboured *L. monocytogenes* (Loncarevic *et al.*, 1996a). The first fish borne outbreak of listeriosis in the world that could be linked to vacuum-packed rainbow trout occurred in 1994–1995 in Sweden. Six non-pregnant cases were immunosuppressed and three were pregnant. All rainbow trout fish isolates belonged to serovar 4b but different PFGE types (Ericsson *et al.*, 1997).

In 2000 in Sweden, 14 vacuum-packed gravad and cold-smoked salmon/rainbow trout samples were collected from 9 different brands. Four samples (29%) from three processing plants harboured *L. monocytogenes* and one sample contained 1600 cfu/g. In total, 60 fish isolates (four isolates of positive samples and isolates submitted by national laboratories from five different plants) were characterised with PFGE. Most of the fish isolates shared serogroup 1/2 and PFGE identified 17 *AscI* types. The most contaminated sample with 1600 cfu/g harboured at least three PFGE types, and encountered in two plants. One PFGE type was found in three plants. A comparison of the fish PFGE types with human *L. monocytogenes* isolates collected during the same year found the three PFGE types in the most heavily contaminated fish sample were common among human isolates (Netterby, 2000). In autumn 2002, the prevalence of *L. monocytogenes* in vacuum-packed gravad, cold-smoked salmon was analysed; 36 fish products were purchased in Malmö and Lund, two in Stockholm and two in Uppsala. Ten of the samples harboured the pathogen and all isolates shared serovar 1/2a and belonged to eight different *AscI* PFGE types that were all identified among human isolates. In six salmon samples from the same manufacturer, a common human PFGE type, with 2000–4000 cfu/g, was identified. One gravad salmon sample harboured three PFGE types and one cold-smoked salmon two PFGE types (Mandorf, 2003).

In 2001 and 2010, the National Food Administration of Sweden requested two large national samplings of food products for analysis of *L. monocytogenes* in RTE foods (Rosengren, 2001; Rosengren & Lindblad, 2003; Thisted Lambertz *et al.*, 2012). In the first national study, including 3439 samples, the presence of *L. monocytogenes* was higher in cold-smoked and gravad fish than in other product groups: gravad fish products (13.8%: 17/123), cold-smoked (7.2%: 6/82) and hot smoked fish (5.5%: 4/73) (Rosengren & Lindblad, 2003). In the second national study, which was part of an EU-wide baseline survey, 1590 RTE foods were sampled throughout the country (packaged gravad fish, hot-smoked fish, cold-smoked fish, soft cheeses, semisoft cheeses, and heat-treated meat products). Of 525 cheese samples, 2 (0.4%) tested positive for the presence of *L. monocytogenes* and among 507 heat-treated meat-products, 5 (1%) was positive. Of 558 fish samples, 66 (12%) tested positive and 14% of both gravad and cold-smoked fish samples was positive. The National Food Administration of Sweden concludes these products constitute the main problem of human listeriosis in Sweden (Thisted Lambertz *et al.*, 2012).

In 2013, the National Food Administration of Sweden, presented results from a large study where isolates of *L. monocytogenes* obtained from human cases during 2005–2010 (183 isolates) were compared with isolates recovered in 2010 from RTE foods (73 isolates) and from 22 meat processing plants (26 isolates), three fish processing plants (5 isolates). However, the pathogen could not be isolated from six cheese-processing plants and one of the fish plants was not located in Sweden. Serovar 1/2a was dominant in all sources, *i.e.* 134 isolates (73%) in human, 70 isolates (96%) in food, and 27 isolates (87%) in the environment; whereas, serovar 4b was less common, 36 (20%) among human isolates, and only one food isolate, isolated from soft cheese. PFGE with *AscI* and *Apal* enzymes identified 94 pulsotypes among 287 isolates. Six unique pulsotypes were identified among human isolates (42%) and fish isolates (59%). The most common human pulsotype is also the most common among fish isolates, and is found only in fish samples from non-Swedish plants (Thisted Lambertz *et al.*, 2013). Serovar 1/2a is the serovar regularly found in cold-smoked and gravad salmon bought in Sweden (Thisted Lambertz

*et al.*, 2013). PFGE types found in human cases of invasive listeriosis in Sweden are frequently encountered in vacuum-packed cold-smoked and gravad salmon/rainbow trout (Netterby, 2000; Mandorf, 2003; Kannius & Karlsson, 2010; Thisted Lambertz *et al.*, 2013).

Listeriosis is still a topical foodborne disease. The world's population is ageing and has a greater chance of developing debilitating chronic conditions. The demographic shift and the widespread use of immunosuppressive medications have increased the immunocompromised population that are susceptible to an increased risk for listeriosis (Allerberger & Wagner, 2010; Lubber *et al.*, 2011; Todd & Notermans, 2011). Consumer lifestyle has changed and less time is available for food preparation. Modern lifestyle has markedly changed eating habits worldwide, with a consequent increased demand for RTE foods (de Oliveira *et al.*, 2010); therefore, more RTE and take away foods are consumed. Within the food industry, extended shelf life RTE foods and new RTE food types are an area of expansion, and as these foods pose the most concern for listeriosis, this area deserves more attention (Allerberger & Wagner, 2010). To be able to control *L. monocytogenes* in RTE products at risk, these products should be properly labeled with regard to time and temperature of storage, and consumers should be educated about food storage practices. In addition, the shelf life for some products with a known risk of *L. monocytogenes* contamination and growth (*e.g.* vacuum-packed gravad and cold-smoked salmon) should be restricted by authority regulations.

There is a concern that many *Listeria* outbreaks are reported from hospitals. Even low levels of *L. monocytogenes* pose a risk to immunocompromised people and RTE food is often served in hospitals. In the United Kingdom, it is recommended that food served to hospital patients is free from *L. monocytogenes* (Fretz *et al.* 2010; Coetzee *et al.* 2011; Yde *et al.*, 2012). Large-scale delivery of precooked meals to a vulnerable population represents a threat if proper measures against listeriosis are not taken (Smith *et al.*, 2011). It is vitally important that foods (especially cooked and chilled) delivered to hospitals, residential homes for senior citizens and elderly people are reheated to at least 72°C and that cold food, such as turkey deli meat and cold-smoked and gravad salmon should be free from *L. monocytogenes*.

Characterisation of *L. monocytogenes* isolates from human patients and foods with different methods has contributed to the identification of human listeriosis globally. Recently, whole genome sequencing (WGS) became a new molecular method for characterisation of clinical *L. monocytogenes* isolates in Sweden (SVA, 2014 and 2015).

### 3 AIMS OF THE THESIS

The main objective of the present work was to improve knowledge of the spread and prevalence of *L. monocytogenes* in humans and foods worldwide, and to elucidate the genetic diversity and relatedness of *L. monocytogenes* isolates in an extensive investigation to provide avenues for future research in this area. To achieve this aim, pulsed-field gel electrophoresis (PFGE) was used as a molecular tool for examining infectious routes and infectious sources of *L. monocytogenes* and for investigating any structure in the occurrence of PFGE types.

The specific aims of the thesis were:

1. to study infectious routes and infectious sources of *L. monocytogenes* through comparison of isolates from human cases and foods (Papers I and IV).
2. to explore the serovar diversity of *L. monocytogenes* isolates from human clinical cases over half a century (Paper I).
3. to investigate, through PFGE with *AscI* restriction enzyme, the genetic diversity of *L. monocytogenes* isolates from human clinical cases of invasive listeriosis in Sweden (Papers I and V).
4. to detect possible clustering of *L. monocytogenes* PFGE types in time or location during half a century in Sweden (Paper I).
5. to analyse the configuration of small bands with sizes < 145.5 kb (PFGE group) of *L. monocytogenes AscI* PFGE profiles, in order to establish the genetic relatedness of *L. monocytogenes* strains isolated from human cases (Papers II and III).
6. to determine whether there is any association between serovars, PFGE types and PFGE groups (Papers I, II and III).
7. to develop a new procedure for identifying *L. monocytogenes* profiles based on the configuration of detectable small bands of sizes < 145.5 kb, and in a later step, the larger band > 145.5 kb, in order to simplify and accelerate the analysis and interpretation of bacterial genomes and identify the source of infection faster without delay (Papers II and III).

## 4 MATERIAL AND METHODS

### 4.1 Bacterial isolates

The clinical and food isolates of *L. monocytogenes* used in the study (collected between 1958 and 2010) are described below.

#### 4.1.1 Human isolates

*L. monocytogenes* isolates from 932 cases (mother-infant is represented by one isolate only) of invasive listeriosis in Sweden were collected between 1958 and 2010. The isolates were stored in brain-heart infusion broth (BHI broth, Merck, Darmstadt, Germany) with 20% glycerol at -73°C. The isolates were obtained from the Swedish Institute for Infectious Disease Control and from different county hospitals around Sweden. Most isolates were isolated from blood and cerebrospinal fluid, with a few from other sterile sites (Papers I, II, III and V).

#### 4.1.2 Food isolates

Fifty-three samples of ready-to-eat, vacuum-packed salmon products were collected from retail stores in Sweden and a further three samples purchased in Germany during summer 2005. The samples obtained in Sweden were from 15 manufacturers and those bought in Germany from one manufacturer. The samples were analysed the day before the best-before date. Altogether, 56 *L. monocytogenes* isolates were isolated from 11 (three manufacturers) of 56 samples (Paper IV).

### 4.2 Isolation and detection methods for *L. monocytogenes*

The isolation and enrichment methods of the Nordic Committee on Food Analysis (NMKL, 2004) were used with some modifications (Paper IV).

#### *Enrichment and culture*

From each food sample, 25g was mashed in a stomacher bag for a few minutes together with 225 ml enrichment broth I. The homogenate was incubated at 30°C for 24 hours. Then, 1 ml of the enrichment culture was added to 10 ml enrichment broth II, with a higher concentration of antibiotics, and incubated for a further 24 hours at 37°C. After incubation, 0.1 ml of enrichment broth II was streaked onto both PALCAM and Oxford agar media. Both plates were incubated at 37°C for 24–48 hours, after which typical *Listeria* colonies were selected for further examination.

#### *Quantification*

All samples were stored at -20°C awaiting the enrichment procedure. From each *L. monocytogenes* positive sample, 10g was mashed in a stomacher bag together with 90 ml of peptone water. Tenfold serial dilutions of 1 ml were made from the suspension and then 0.1 ml was streaked onto blood, PALCAM and Oxford agar media. All plates were incubated for 48

hours at 37°C, after which all typical *L. monocytogenes* colonies were counted. Five colonies taken from both the enrichment and the quantification plates were streaked onto blood agar for purity.

#### *Confirmation*

Five typical colonies chosen from each plate were re-streaked onto blood agar and tested for Gram reaction, rhamnose and xylose fermentation. Motility test was in BHI broth incubated for 10 hours at 20°C.

### 4.3 Conventional serotyping of *L. monocytogenes*

Serotyping was with commercial O-antigen *Listeria* antisera types I/II, I, IV, V/VI, VI, VII, VIII, IX and H-antigen *Listeria* antisera types A, AB, C, D (Mast Diagnostics, Mast house, Berby Road, Bootle, Liverpool, UK 201EA), according to the manufacturer's instructions, with some modifications (Papers I and IV). Isolates from each of the freeze-stored bacterial cultures were streaked onto horse blood agar plate with a sterile plastic loop and incubated at 37°C for 24 h. Then, 3 ml of 0.2% sodium chloride was added to the bacterial growth and mixed with a glass spatula. The suspension was transferred to a tube and boiled in a water bath at 100°C for 1h, left at room temperature for 30 min before being centrifuged at 3700 rpm for 20 min. After resuspension in 1 ml of 0.2% sodium chloride, the pellet was tested for agglutination with the O antiserum, according to manufacturer's instructions. To determine the H antigens, the isolates of each freeze-stored bacterial culture were streaked onto horse blood agar plate with a sterile plastic loop, and then incubated at 37°C for 24 h. The cultures were passed three times through semi-solid nutrient agar plates at 30°C for 24 h. Colonies removed from the plate were inoculated into 2ml of BHI broth with a sterile plastic loop and incubated at 30°C for 24 h. Then, 2 ml of 0.9% sodium chloride was added and the suspension kept at 30°C overnight. The tube agglutination test was with H antiserum and the prepared cell suspension, according to the manufacturer's instructions.

### 4.4 Molecular typing by pulsed-field gel electrophoresis (PFGE) of *L. monocytogenes*

Each isolate was characterised by PFGE and restricted with *AscI* enzyme according to the Pulse Net standardised protocol described by Graves and Swaminathan (2001). Only 11 of 56 food isolates were analysed with *ApaI* restriction enzyme (Paper IV). Through serovars and *AscI* profiles, different PFGE types of *L. monocytogenes* were obtained (Paper I and IV). For each type, a reference strain was selected (Paper I). The protocol (Graves & Swaminathan, 2001) used was modified as follows.

#### 4.4.1 Preparation of gel plugs

The isolates of each freeze-stored bacterial culture were streaked onto horse blood agar plates with a sterile plastic loop and incubated at 37°C for 24 h. One well isolated colony was removed



from the plate and inoculated into 5 ml sterile BHI broth (Oxoid CM 225) with a sterile plastic loop and then incubated in a water bath at 37°C for 24 h. The tube was vortexed for 3 seconds and kept in a water bath at 4–8°C for 5–10 min. After centrifugation at 5700 rpm for 5 min, 4.5 ml of the supernatant was discarded and the pellet was vortexed to a homogeneous suspension. Thereafter, 5 ml of cold TN buffer (10 ml 1 M TrisHCl, pH 8; 20 ml 5 M NaCl and 970 ml sterile water) was added and the sample vortexed for 5 seconds. The suspension was cooled and kept in a water bath at 4–8°C for 5–10 min and centrifuged at 5700 rpm for 5 min. Then 4.5 ml of the supernatant was discarded and the pellet was vortexed to a homogeneous suspension. The bacterial suspension was treated with 0.9 ml of lysozyme, Roche (1mg lysozyme/ 1ml TN buffer), vortexed 3 seconds and incubated in a water bath at 37°C for 30 min. Fifteen ml of 1.2% agarose solution (180 g SeaKem Gold agarose, Cambrex, and 15 ml sterile water) was prepared and kept in a water bath at 55°C for 10 min. Then 1.67 ml of ESP buffer (1g N-lauroylsarcosine, Merck; 100 ml 0.5 M EDTA, pH 8 and 200 mg pronase, Roche) was added to the agarose solution and vortexed for a few seconds. The bacterial suspension with lysozyme was transferred to the water bath at 55°C, then mixed with 1.2 ml of agarose solution, vortexed for 3 seconds and incubated in a water bath at 55°C for 30 min. The mixture was poured into three wells of a plug mould (Gene Navigator Pharmacia, Biotech, Uppsala, Sweden) with a sterile Pauster pipette and placed in the refrigerator for 10 min. The gel plugs were transferred to a sterile microcentrifuge tube containing 1 ml of ESP buffer and kept in a water bath at 55°C for 1 h, after which, the ESP buffer was renewed and the test tube was incubated in a water bath at 55°C for further 24 h. The ESP buffer was renewed twice during two days and the gel plugs were then stored in the refrigerator until ready for restriction.

#### 4.4.2 Restriction digestion of DNA

All isolates were restricted with *AscI* restriction enzyme (New England Biolabs, Inc.). A plug slice, approximately 1.5 mm was placed into a micro centrifuge tube containing 0.5 ml of PEFA solution (3.5 mg Pefablock, Roche, and 10 ml TE buffer [10 ml 1 M Tris HCl, pH 8; 2 ml 0.5 M EDTA, pH 8 and 1000 ml sterile water]) and incubated in a water bath at 37°C for 40 min. After which, the PEFA solution was renewed and the micro centrifuge tube was incubated in a water bath at 37°C for a further 40 min. The solution was aspirated and replaced by 1 ml TE buffer and incubated twice during 40 min in a water bath at 55°C. Then TE buffer was aspirated and replaced by 150 µl restriction solution (870 µl sterile water; 108 µl 10x NE4 buffer, New England Biolabs, Inc; 10 µl 10 mg/ml BSA, Promega, and 12 µl 10 U/µl *AscI*) and incubated at 37°C for 15–18 h. The isolates restricted with *ApaI* restriction solution (870 µl sterile water; 98 µl 10x buffer A, Boeringer Mannheim; 10 µl 1 mg/ml BSA, Promega, and 22 µl 10 U/µl *ApaI*, Boeringer Mannheim) and incubated at 30°C for 15 h.

#### 4.4.3 Electrophoresis

The restriction solution was aspirated and replaced by 200 µl of 0.5 x TBE [9 ml sterile water and 1 ml 5 x TBE buffer (54 g 0.45M Trisbas, Amersham Biosciences; 27.5 g 0.45 M Boric acid, Merck; 20 ml 0.5 M EDTA, pH 8 and sterile water up to 1000 ml)] and incubated at 37°C for 30–60 min. Then, 1.2% agarose solution was prepared (1.3 g SeaKem Gold agarose, 11 ml 5 x TBE

buffer and 99 ml sterile water) and kept in a water bath at 55°C. The agarose solution for plugs restricted with *ApaI* was 1% (1.4 g SeaKem Gold agarose, 14 ml 5 x TBE buffer and 126 ml sterile water). The plug slice was removed carefully from the microcentrifuge tube and loaded onto comb teeth, and then the comb was transferred to a gel form. The plug was pushed gently to the bottom and covered with a dash of agarose. The remaining 1.2% agarose solution was poured into the gel form and left at room temperature for 15 min. The comb was removed from the gel and the wells were filled with agarose. A Lambda ladder PFGE Marker NO340S (New England Biolabs, Inc.) was used as molecular weight standard (50 to 1,000 kb). A Pharmacia Gene Navigator (Pharmacia LKB Biotechnology AB, Uppsala, Sweden) was used to separate DNA fragments in plugs restricted with *AscI* under the appropriate electrophoretic parameters: migration period was 24 h at 200 V: initial switch time 4.0 sec, final switch time 40.0 sec, and running buffer 0.5 x TBE at 8°C. The plugs restricted with *ApaI* were separated by electrophoresis in 0.5 x TBE at 14°C in CHEF MAPPER XA (BIO-RAD), run time being 20 h with initial switch time of 1.0 sec and final switch time of 15.0 sec. After electrophoresis, the gel was stained for 25 min in 1 L 0.5 x TBE buffer containing 100 µl ethidium bromide, Molbase (1 mg/ml) and then destained for 1 h in 1 L 0.5 x TBE buffer. Then, the banding patterns of the gels were visualised with short-wave ultra violet (312 nm) light and photographed with a Polaroid camera.

#### 4.4.4 Analysis of PFGE profiles

The DNA profiles were analysed visually and the position of each restriction fragments of each PFGE profile was sized with both Lambda ladder and the universal marker *Salmonella* Braenderup H9812 (digested with *XbaI*, Roche) to establish the reference position. PFGE types were established based on the number and distribution of all detectable fragments in a DNA restriction profile (Paper I and IV), whereas, PFGE groups were established solely on the number and distribution of all detectable small fragments below 145.5 kb (Papers II and III). PFGE profiles were considered distinguishable if the banding patterns were different. PFGE profiles with identical banding patterns below 145.5 kb were considered as sharing the same PFGE group. The reference collection was created by selecting a reference profile from each PFGE type. The new reference standard profile was designated by a numerical code, for example the designation A:1/2a:4A indicated the strain belonged to PFGE group A, serovar 1/2a and represented the 4th *AscI* profile among the serogroup 1/2 isolates of the collection. The last letter indicates the *AscI* profile no 4 has various closely-related variants, in this case the strain is variant A (Tenover *et al.*, 1995).

#### 4.5 In silico analyses

Thirteen completely sequenced *L. monocytogenes* genomes belonging to lineage I (serovar 1/2b, 3b, 4b and 4e), and lineage II (serovar 1/2a, 1/2c, 3a and 3c) were obtained from either website insilico.ehu.es, or EMBL-EBL. Each sequenced genome was analysed *in silico* with PFGE and restriction enzyme *AscI*. The *in silico* program calculated the theoretical number of restriction fragments in the DNA (Papers II and III).

## 5 RESULTS AND DISCUSSION

### 5.1 Serovars of *L. monocytogenes*

The study period in Sweden, 1958–2010, could be divided into three periods: 1958–1971, where three serovars 1/2a, 1/2b and 4b were equally common; 1972–1995, where serovar 4b was prevalent; and, 1996–2010 where serovar 1/2a was the major *L. monocytogenes* serovar in human listeriosis in Sweden (Paper I: Table 2). The three periods are discussed below.

#### 5.1.1 Human *L. monocytogenes* isolates from 1958–1971 in Sweden

Since Linell presented the first case of human listeriosis in Sweden in May 1958 (Linell *et al.*, 1959), a limited number of cases were reported in Sweden, as it was difficult to diagnose the disease (Meyer, 1961; Sepp & Roy, 1963; Seeliger & Finger, 1983). In the UK, McLauchlin (1990b) observed a similar pattern during 1967–1985 and considered improvement in isolation and identification of the pathogen influenced the reporting of the cases. The majority of listeriosis cases reported in the 1950s and 1960s in Sweden were associated with pregnancy; a trend also seen in other countries (Ekelund *et al.*, 1962; Sepp & Roy, 1963; Seeliger *et al.*, 1969; Becroft, *et al.*, 1971; Guevara *et al.*, 1979; Campbell, 1990; McLauchlin, 1990b). During the 1970s, between 10 and 15 human listeriosis cases were reported annually in Sweden; however, there is a lack of food isolates of *L. monocytogenes* available from this time. From 1958 to 1971, the three *L. monocytogenes* serovars 1/2a, 1/2b and 4b were equally common among human isolates, although based on a small number of available isolates (Paper I). Serovar 1/2a predominated in West Germany until 1959, whereas, from 1960 most human strains were serovar 4b (Seeliger *et al.*, 1969).

#### 5.1.2 Human *L. monocytogenes* isolates from 1972–1995 in Sweden

In the period 1972–1995, serovar 4b was the most common serovar found in human cases of listeriosis in Sweden (58.1% of available isolates), and 32.5% of isolates shared serovar 1/2a. During the 1970s, 1980s, and 1990s, the majority of human cases of listeriosis worldwide, were linked to lineage I serotype 4b *L. monocytogenes* isolates (Guevara *et al.*, 1979; Campbell, 1990; Kathariou, 2002; Paper I). At the same time, *L. monocytogenes* serovar 4b was a common finding in soft and semi-soft cheeses in Europe (Ericsson *et al.*, 1996). French cheeses purchased in Swedish retail outlets during 1987 and 1993 harboured *L. monocytogenes* serovar 4b, and two smear cheeses contained 2300 and 100,000 cfu per g (Danielsson-Tham, 1993; Loncarevic *et al.*, 1995).

#### 5.1.3 Human *L. monocytogenes* isolates from 1996–2010 in Sweden

Among the available human *L. monocytogenes* isolates during this period, serovar 1/2a was predominant (68.7%) (Paper I), probably due to increased consumption of gravad and cold-

smoked fish as *L. monocytogenes* serovar 1/2a is common in these products (Paper IV). This observation is in agreement with other studies from Sweden (Loncarevic *et al.*, 1996b; Rosengren, 2001; Rosengren & Lindblad, 2003; Thisted Lambertz *et al.*, 2012; Thisted Lambertz *et al.*, 2013). The decrease in cases of listeriosis caused by serovar 4b strains since 1996 in Sweden may be due to the measures taken at the RTE food level in France and other European countries. During a study from May-December 1999, Rudolf and Scherer (2001) report an improvement in processing hygiene, as only 4 (4.4%) out of 91 French soft raw milk cheeses (red smear) were positive for *L. monocytogenes*. However, the overall results from analyses of 329 European soft, semi-soft and hard red smear cheeses made from raw or pasteurised milk were not encouraging; 6.4% were positive for *L. monocytogenes* (Rudolf & Scherer, 2001). Thus, red smear cheeses should still be regarded as a considerable public health risk. In 2005, 12.9% of RTE vacuum-packed gravad salmon and 28% of cold-smoked salmon harboured *L. monocytogenes*: the highest level of *L. monocytogenes* was 1500 cfu/g from a cold-smoked salmon product (Paper IV). The most common PFGE types found in human cases of listeriosis in Sweden are also the types frequently encountered in vacuum-packed cold-smoked and gravad salmon/rainbow trout (Netterby, 2000; Mandorf *et al.*, 2003). Since the early 2000s, the incidence of listeriosis has increased in Europe (SVA, 2007; Little *et al.*, 2012).

#### 5.1.4 The shift from serovar 4b to serovar 1/2a in human listeriosis

A shift from *L. monocytogenes* serovar 4b to serovar 1/2a occurred in the middle of the 1990s and the early 2000s in both Sweden and Europe. In 1987, 59% of 722 cases of listeriosis in Britain were reported as due to *L. monocytogenes* serovar 4b (McLauchlin, 1987), and in 1991, serovar 4b prevailed in human listeriosis in most of Europe, with serovar 4b accounting for 63.9% in France and 64% in United Kingdom (Farber & Peterkin, 1991). Generally, most food-borne listeriosis outbreaks appear to have been caused by serovar 4b (Buncic *et al.*, 2001; Orsi *et al.*, 2011; Cartwright *et al.*, 2013). However, large outbreaks of listeriosis predominantly caused by serogroup 4b strains have become less frequent (Swaminathan & Gerener-Smidt, 2007). During an international Listeria conference (ISOPOL) in Australia, Gerner-Smidt *et al.* (1995) reported that until 1992 in Denmark, two-thirds of human *L. monocytogenes* isolates belonged to serogroup 4: the following year, 1993, serogroup 1 became predominant in Denmark. McLauchlin and Newton (1995) reported similar results from United Kingdom at the same conference and conclude that “The trends towards an increase in listeriosis amongst the immunocompromised, together with the increase in cases due to serogroup 1/2 mean that future resources will be directed at improving our ability to subtype within serogroup 1/2” (McLauchlin & Newton, 1995). The Danish and the British conference abstracts from 1995 were among the first reports of a shift from *L. monocytogenes* serogroup 4 to serogroup 1 in human listeriosis. In Sweden, serogroups 4 and 1/2 were equally common during the decade 1986 to 1996 (Loncarevic *et al.*, 1998b); however, “the changes in serogroup distribution among human cases in Sweden ... might be explained by changes in eating habits” (Loncarevic *et al.*, 1998b).

Orsi *et al.* (2011) stress lineage II serotype 1/2a strains appear more common among human listeriosis cases in Northern Europe; which is confirmed by Rosef *et al.* (2012) from Norway, where 55.6% of human clinical isolates collected between 1992 and 2005 were lineage II, and 41.6% were lineage I with ribotyping. The change in the serotypes of human *L. monocytogenes*

isolates has also been observed in Finland in *L. monocytogenes* isolates from invasive infections during an 11-year period, 1990–2001. Since 1990, the number of cases caused by serotype 4b has remained constant, with some exceptions. However, the number of listeriosis cases caused by serovar 1/2a increased during 1990–2001 and exceeded the number of cases caused by serovar 4b since 1991 (Lukinmaa *et al.*, 2003). Even during 2002–2004, serovar 1/2a was predominant in Finland (Lyytikäinen *et al.*, 2006). In Denmark, during 2002–2012, 42% of human isolates belonged to lineage I and 58% to lineage II: the lineage II isolates belonged to serotype 1, predominantly serovar 1/2a, as determined by PCR (Kvistholm Jensen *et al.*, 2016). Serovar 1/2a has been the dominant *L. monocytogenes* serovar in human listeriosis in Sweden since 1996 (Paper I).

The shift from serovar 4b to 1/2a is also seen in Switzerland, serogroup 1/2 became predominant among human strains after 1994 (Pak *et al.*, 2002), and during 2011–2013, the number of human isolates of serovar 1/2a was more than twice the number of 4b isolates (Althaus *et al.*, 2014). During 1990–1999, serovar 4b dominated (52%) among human strains in different regions in Italy, whereas, serovar 1/2a constituted only 27.2%. However, during 2002–2005, serovar 1/2a constituted 50.9% and serovar 4b 24.6% of human strains (Gianfranceschi *et al.*, 2003, 2009). Serovar 1/2a was also the predominant serovar in the Lombardy region, Italy, between 2006 and 2010 (Mammìna *et al.*, 2013). Mammìna *et al.* (2013) suggest serotype 1/2a has replaced serotype 4b worldwide as the leading serotype causing human listeriosis. In Canada, between 1995 and 2004, serotype 1/2a was the predominant serotype among human cases, with serotype 4b being predominant in cases associated with pregnancy and miscarriage. Among 722 isolates, serovar 1/2a (47.5%) and serovar 4b (30%) were identified (Clark *et al.*, 2010); however, there is no evidence that lineage II strains are common in human listeriosis outbreaks (den Bakker *et al.*, 2010).

Several countries have experienced a shift from *L. monocytogenes* serovar 4b to 1/2a in causing human infections. The increase in the incidence of human listeriosis among patients > 60 years old in England and Wales between 2001 and 2007 appears to be associated with cancer or other conditions requiring treatment to reduce stomach acid secretion (Gillespie *et al.*, 2009). Swaminathan & Gerner-Smidt (2007) claim blood stream infection (bacteraemia) is now a more common clinical presentation in listeriosis than meningoencephalitis and serovars 1/2a and 1/2b are more common in blood stream infections than in meningoencephalitis. Thus, more blood stream infection will lead to more serovar 1/2a and 1/2b in listeriosis. Swaminathan & Gerner-Smidt (2007) suggest “the chance a blood stream infection will be detected has increased because the blood culturing systems have become more sensitive and the indications for drawing a blood culture have become broader the past 20 years.”

A simple reason for more 1/2a cases of listeriosis may be due to the frequent occurrence of this serovar in the increasingly popular ready-to-eat foods (Buncic *et al.*, 2001; Gilbreth *et al.*, 2005, Paper I). A link between isolates obtained from patients and isolates obtained from smoked fish is reported in Scandinavian countries (Sweden, Norway, and Finland) and in eastern Spain (Luber *et al.*, 2011; Ariza-Miguel *et al.*, 2015). In the 2000s, several outbreaks where serovar 1/2a was involved are reported: fresh cheese (Danielsson-Tham, 2004), sliced processed delicatessen turkey meat (Olsen *et al.*, 2005), flat whipping cream (Pagotto *et al.*, 2006), tomme cheese (Bille *et al.*, 2006), Quargel-acid curd cheese (Fretz *et al.*, 2010), delicatessen meats

(Garrido *et al.*, 2010; Farber *et al.*, 2011), and soft washed-rind cheese (Gaulin *et al.*, 2012). Furthermore, later outbreaks of listeriosis are linked to *L. monocytogenes* serotype 1/2a (Tham and Danielsson-Tham, 2014; Ariza-Miguel *et al.*, 2015; Public Health Agency of Sweden, 2017).

Although serovar 4b has previously been predominant, since the middle of the 1990s, there has been a shift in human listeriosis in Sweden and currently *L. monocytogenes* serovar 1/2a isolates are more frequently isolated than serovar 4b (SVA, 2011–2015; Personal communication: Hedemström, 2017)

## 5.2 Seasonal variation

From 932 human *L. monocytogenes* isolates from 1958–2010, the majority of serovar 1/2a isolates (29.5%) were received during October to January, whereas, the majority of serovar 4b and 1/2b isolates (32.6%) were received during June to October (Paper I). This observation was in agreement with a study in the USA (Orsi *et al.* (2011), where more human serovar 1/2a isolates were found in water during autumn and winter, whereas, human serovar 4b was more often isolated during summer. In contrast, an Iranian study reported that among seafood products and environmental isolates (Fallah *et al.*, 2013), serovar 1/2a isolates are dominant in warm periods and serovar 4b during cold seasons. Between 1967 and 1985, the seasonal peaks of human listeriosis in the UK and Scotland were in the late summer and autumn (Campbell, 1990; McLauchlin 1990a; McLauchlin 1990b) and *Listeria* bacteria were frequently isolated in faeces and soils during July to September (MacGowan *et al.*, 1994). No significant seasonal variations were observed in Latvia during 1998–2007, except for a peak of listeriosis during September–December, 2000 (Bērzinš *et al.*, 2009).

In Sweden, most pregnancy-associated isolates (n=27, 27.6%) arrived at the laboratory during summer (July–Sept) and the least (n=16, 16.3%) during autumn (Oct–Dec: Table 4). In the north of Sweden (Jämtland, Västerbotten and Norrbotten), cases of listeriosis are registered almost entirely during January–March, whereas, in the south of Sweden (Skåne), the majority of cases are registered during summer and autumn. According to Danish researchers, the prevalence of *L. monocytogenes* is higher during the summer due to the increase in production that hinders control measures in the meat processing plants. A similar trend has been observed in fish processing plants, although during November and December (Tompkin *et al.*, 1992).

Table 4. Serovars and seasonal distribution of *Listeria monocytogenes* isolates in pregnancy-associated cases in Sweden, per decade, between 1958 and 2010.

Period	Isolates (n)*	Pregnant/ child		Serovar			Season				
		(n)	%**	1/2a	1/2b	4b	Winter	Spring	Summer	Autumn	no data
1958–1969	27	7	25.9	0	2	4	1	1	1	1	3
1970–1979	101	33	32.7	2	1	30	6	8	6	6	7
1980–1989	169	22	13.1	5	1	16	2	3	7	5	5
1990–1999	219	22	10.0	6	1	15	4	5	8	2	3
2000–2010	416	18	4.3	11	2	5	6	4	5	2	1
Total	932	102		24	7	70	19	21	27	16	19

\*Non-pregnant and pregnancy-associated isolates. \*\*Percentage of pregnancy-associated cases

### 5.3 Geographical variation

All *L. monocytogenes* isolates of human cases come from the three regions of Sweden: Götaland (Southern: n=526, 56%), Svealand (Central: n=294, 32%) and Norrland (Northern: n=108, 12%). The geographical distribution indicated the majority of cases of listeriosis occurred in Southern of Sweden, where most of the cases were from Skåne (n=225, 24%), Västra Götaland, in the southwest (n=173, 19%) and Stockholm in the central region (n=141, 15%). In Västernorrland county there were less cases of listeriosis (n=56, 6%) (Table 5). Most cases occurred in the cities of Stockholm (15%), Göteborg (13%), Malmö (11%) and Lund (8%): the differences in percentage appear related to population density of the counties.

**Table 5.** Numbers and serovars of human *Listeria monocytogenes* isolates in Sweden, per county, during 1958–2010.

Counties	Population 2011* (%)	Isolates (n)	Serovars										
			%	1/2a	%	1/2b	%	1/2c	%	4b	%	3b	%
Skåne	1,252,933 (13.2)	225	24.1	106	21.9	28	31.1	5	23.8	85	25.3	1	33.3
Blekinge	152,979 (1.6)	13	1.4	7	1.4	1	1.1	2	9.5	3	0.9	0	0.0
Halland	301,724 (3.2)	30	3.2	20	4.1	1	1.1	2	9.5	6	1.8	1	33.3
Västra Götaland	1,590,604 (16.8)	173	18.6	76	15.7	17	19.0	3	19.0	76	22.6	1	33.3
Gotland	57,308 (0.6)	8	0.9	6	1.2	0	0.0	0	0.0	2	0.6	0	0.0
Kronoberg	184,654 (1.9)	17	1.8	10	2.1	1	1.1	0	0.0	6	1.8	0	0.0
Kalmar	233,090 (2.5)	19	2.1	11	2.3	1	1.1	1	4.8	6	1.8	0	0.0
Jönköping	337,896 (3.6)	14	1.5	9	2.0	1	1.1	0	0.0	4	1.2	0	0.0
Östergötland	431,075 (4.5)	27	2.9	10	2.1	8	9.0	0	0.0	9	2.7	0	0.0
Örebro	281,572 (3.0)	12	1.3	6	1.2	1	1.1	0	0.0	5	1.5	0	0.0
Södermanland	272,563 (2.9)	13	1.4	7	1.4	2	2.2	0	0.0	4	1.2	0	0.0
Västmanland	254,257 (2.7)	34	3.6	16	3.3	2	2.2	2	9.5	14	4.2	0	0.0
Uppsala	338,630 (3.6)	32	3.4	18	3.7	4	4.4	1	4.8	9	2.7	0	0.0
Stockholm	2,091,473 (22.1)	141	15.1	79	16.4	10	11.1	2	9.5	50	14.9	0	0.0
Värmland	272,736 (2.9)	26	2.8	11	2.3	1	1.1	0	0.0	14	4.2	0	0.0
Dalarna	276,565 (2.9)	36	4.0	17	3.5	6	6.8	1	4.8	12	3.6	0	0.0
Gävleborg	276,130 (2.9)	17	1.8	12	2.5	1	1.1	0	0.0	4	1.2	0	0.0
Västernorrland	242,155 (2.5)	56	6.0	40	8.3	3	3.3	1	4.8	12	3.6	0	0.0
Jämtland	126,299 (1.3)	4	0.4	3	0.6	0	0.0	0	0.0	1	0.3	0	0.0
Västerbotten	259,667 (2.7)	16	1.7	8	1.7	2	2.2	1	4.8	5	1.5	0	0.0
Norrbotten	248,545 (2.6)	15	1.6	10	2.1	0	0.0	0	0.0	5	1.5	0	0.0
no data	0	4	0.4	1	0.2	0	0.0	0	0.0	3	0.9	0	0.0
Total	9,482,855 (100)	932	100.0	483	100.0	90	100.0	21	100.0	335	100.0	3	100.0



## 5.4 Age and gender

In the early 1960s, Seeliger (1961) reports no particular gender association, except susceptibility to listeriosis during pregnancy, or age correlation. However, females aged 20–49 years were more associated with listeriosis than males in the same age group (Table 5 in Paper I). Although this is a normal pattern due to female association with pregnancy, the incidence of listeriosis in males aged 50–89 years was high (59.4%) and generally listeriosis is more frequent in males in Sweden. A predominance of listeriosis in males is reported from England (56%), Switzerland (58%), France (62%), Israel (64%) and Portugal (62%) (Jones *et al.*, 1994; Büla, 1995; Goulet & Marchetti, 1996; Siegman-Igra *et al.*, 2002; Almeida *et al.*, 2006). The number of cases associated with pregnancy has decreased over time in Sweden (Table 4), as the increased health focus on pregnant women may have reduced exposure to the *Listeria* pathogen. A similar trend is observed in other countries (McLauchlin, 1996b; Antal *et al.*, 2007). Conversely, non-pregnant cases have increased globally. Bertrand *et al.* (2016) observe an increase among non-pregnant cases in Belgium and suggest “probably due to the rise of highly susceptible patients in an aging population...” and “...can be attributed to significant increase in incidence of isolates belonging to serovar 1/2a”. The National Food Administration in Sweden has provided advice on food to pregnant women since the early 1990s through the website and brochures in maternity health care centres. Despite dietary recommendations being translated into other languages, 5 of 7 pregnant cases in 2010 were women with foreign backgrounds (Ivarsson, 2010), and food-safety advice concerning risk food for listeriosis may not reach pregnant women belonging to ethnic minorities (Mook *et al.*, 2010). Although the incidence of listeriosis cases associated with pregnancy has remained stable in Norway and other European countries, there is a clear preponderance of serovar 4b within pregnancy-associated isolates. Among 102 isolates from the period studied, 70 (68.6%) shared 4b, 24 (23.5%) isolates belonged to serovar 1/2a, and 7 (6.9%) to serovar 1/2b: these results presented similar patterns to pregnancy-associated cases reported in other studies (McLauchlin, 1990a; Doorduyn *et al.*, 2006; Goulet *et al.*, 2008; Clark *et al.*, 2010).

Between 2000 and 2010, 75% of the listeriosis cases in Sweden were aged >60 years (Paper I: Tables 5 and 6). In England and Wales between 2001 and 2007, the number of cases in those ≥ 60 years with bacteraemia and without involvement of central nervous system has increased. The increase relates especially to digestive-organ malignancies (cancer) or other underlying conditions and in whom treatment included acid-suppressing medication such as proton pump inhibitors. Patients with CNS infections have fewer underlying conditions than those with bacteraemia (Gillespie *et al.*, 2009; Allerberger & Wagner, 2010). In Denmark, Kvistholm Jensen *et al.* (2016) also report an increase in blood stream infections among patients > 60 years old and during 2005–2009, lineage II isolates were the main cause of the increase. The increased number of listeriosis cases in France 2006–2007, was mainly due to a rise in cases in people > 60 years old, regardless of any underlying condition (Goulet *et al.*, 2008). Serovar 1/2c was prevalent (66.7%) among male isolates, and the association of males with the less-common serovars 1/2c is noted in other studies (Andersson, 1980; Lukinmaa *et al.*, 2003). Andersson (1980) concludes this trend “may be explained by consumption of Steak tartar or other raw meat especially appreciated by males”. Among 133 *L. monocytogenes* isolates from meat products collected in France, serovar 1/2c accounted for 37.6%, whereas, serovar 1/2a was found in 25.6%, serovar 1/2b in 15% and serovar 4b accounted for 12.8% of isolates (Rocourt *et al.*, 1992). In Finland, 5% of 180 male isolates shared one PFGE type belonging to serovar 1/2c

(Lukinmaa *et al.*, 2003). In the catering industry, comprehension about hygiene (kitchen, equipment and personal) is lower among male employees than among female employees (Çakıroğlu & Uçar, 2008). Al-Shabib *et al.*, (2017) observed males have riskier behaviour regarding cleaning kitchens than females; therefore, differences regarding gender and listeriosis can be partly to do with food safety awareness, which is stronger among females (Jevšnik *et al.*, 2008).

## 5.5 Pulsed-field gel electrophoresis (PFGE) types and PFGE groups

A major goal for public health scientists during investigations of food-borne cases and outbreaks is the analysis and interpretation of bacterial genomes to assist in identification of sources and origin of contamination in order to control and prevention measures to be implemented. Therefore, initial screening and comparison of genomes requires simplification. PFGE with restriction enzyme *AscI* is efficient in distinguishing *L. monocytogenes* PFGE groups (based on the number and distribution of all detectable small fragments below 145.5 kb), and PFGE types (based on the number and distribution of all detectable fragments above 145.5 kb). Although *L. monocytogenes* isolates are from different sources, times and places, their small fragments are genetically more conserved than the major fragments. The small fragment configuration reveals which PFGE group, lineage and serovar the isolate belongs to (Papers II and III). The *AscI* PFGE group identifies relatedness of two different PFGE types that belong to a same PFGE group, even if profiles differ more than three bands above 145.5 kb: this is supported by figures (DNA profiles) published by Burall *et al.* (2016), Chen *et al.* (2016b), Chen *et al.* (2017b) and Li *et al.* (2017)

The 63 PFGE types of *L. monocytogenes* identified among 427 clinical isolates belonging to lineage I could be further divided into 17 PFGE groups, based on the position of distinctly detectable bands below 145.5 kb. Among 334 isolates of serovar 4b, 30 PFGE types were identified, which were further divided into 8 PFGE groups (I, J, Q, R, X, Z, Ö-4 and Ö-5). Furthermore, the 90 serovar 1/2b isolates were distributed into 32 PFGE types and nine PFGE groups (D, G, O, P, T, U, Ö-1, Ö-2 and Ö-3). PFGE group G also included a PFGE type belonging to serovar 3b (3 isolates). Five of the 17 PFGE groups were each represented by only one isolate in each group (Ö-1 to Ö-5). Small fragments below 33.3 kb were non-detectable in *L. monocytogenes* lineage I isolates (Paper II): the absence of small fragments is reported in other studies (Brosch *et al.*, 1994; Johansson *et al.*, 1999; Miettinen *et al.*, 1999a).

Altogether 504 clinical isolates belonged to lineage II including 483 serovar 1/2a isolates distributed into 114 PFGE types and 21 serovar 1/2c isolates distributed into 9 PFGE types were further divided into 21 PFGE groups. *L. monocytogenes* isolates belonging to PFGE groups A, B, C, E, F, H, K, L, M, S, V, W, Y, Ö-6 to Ö-12 shared serovar 1/2a, with one exception: PFGE group E also included two PFGE types sharing serovar 1/2c and four PFGE types belonging to either serovar 1/2a or 1/2c. Isolates belonging to PFGE group N shared only serovar 1/2c. Generally, all *L. monocytogenes* isolates displaying one PFGE type belonged to the same serovar. No serovar 1/2a isolates shared restriction profiles with serovar 4b isolates: these observations are corroborated by others (Johansson *et al.*, 1999; Autio *et al.*, 2002;

Gianfranceschi *et al.*, 2009). In contrast to lineage I isolates, small fragments < 33.3 kb were visible in all *L. monocytogenes* isolates belonging to lineage II (Paper III).

The average number of human isolates was 5.1 per PFGE type, *i.e.* 932 human *L. monocytogenes* isolates were distributed into 183 PFGE types. The highest average belonged to serovar 4b (10.8 isolates), whereas, the average serovar 1/2a isolates was 4.2 isolate per PFGE type (Paper I). Among 559 human *L. monocytogenes* isolates collected during 2002–2012 in Denmark, 122 *AscI* profiles were identified, *i.e.* an average of 4.6 isolates per pulsotype (Kvistholm Jensen *et al.*, 2016). In The Netherlands, 134 *L. monocytogenes* isolates were characterised from human cases between 1999 and 2003 and PFGE identified 58 genotypes, an average of 2.3 isolates per genotype (Doorduyn *et al.*, 2006). In Finland, 81 *AscI* PFGE were identified from 314 human *L. monocytogenes* isolates, an average of 3.9 isolates per PFGE type (Lukinmaa *et al.*, 2003). In Switzerland, 93 *L. monocytogenes* isolates were analysed from human cases collected between 2011 and 2013 (Althaus *et al.*, 2014): PFGE yielded 70 different *AscI* types, an average of 1.3 isolates per PFGE type. The authors conclude “the population of Swiss *L. monocytogenes* strains is representative of the known diversity of this species and demonstrates the presence of all major *L. monocytogenes* clones in Switzerland” (Althaus *et al.*, 2014). The average of isolates per PFGE type among 932 human *L. monocytogenes* isolates (Paper I) indicated this species is genetically heterogeneous, which was in concordance with other studies (Cantinelli *et al.*, 2013; Haase *et al.*, 2014).

The highest diversity of PFGE types was identified in four counties: Skåne 79 of 183 types (43%), Västra Götaland (72 types (39%)), Stockholm (64 types (35%)) and Västernorrland (29 types (16%)) types. One of the most common PFGE types in Sweden is A:1/2a:4A (58 cases), was first identified in 1982, and was still isolated at the end of the study period in 2010, with 11 human cases. This type was detected in 17 counties, but mainly in Skåne (9 cases), Västra Götaland (11 cases), Stockholm (9 cases) and Västernorrland (8 cases). In contrast, the closely related type A:1/2a:4B (40 cases) was identified later in 1988 and was isolated from six human cases at the end of study period. This type was detected in 14 counties, mostly in Skåne (15 cases: Table 6). Both types, A:1/2a:4A and A:1/2a:4B, were not present at the beginning of the study period, but appeared in the early 1980s. Similarly, in Canada, human isolates sharing subtype LMACI.0009:LMAAI.0234 serovar 1/2a were not identified before 2002, but have been frequently isolated since 2002 (Clark *et al.*, 2010). The two closely related PFGE types I:4b:3 (first isolated in 1976) and I:4b:6 (first isolated in 1971) were the most common types in the study period with 67 cases each. Both PFGE types were isolated from 1 (I:4b:3) and 5 (I:4b:6) human cases in 2010 (Papers I and II) and were detected in 13 (I:4b:3) and 11 (I:4b:6) counties, mostly in Skåne and Västra Götaland (Table 6). PFGE type J:4b:5 (64 cases) was present during a long period (1965–22008) and was found in 16 counties, mostly in Skåne (14 cases) and Västra Götaland (16 cases: Table 6). PFGE type E:1/2a:9B (36 cases) was first identified in 1993 (1 case) and in 2010 (8 cases) and was detected in 12 counties, mainly in Skåne (8 cases) and Västernorrland (7 cases). The population structure of *L. monocytogenes* was mainly clonal and stable over time and space. Furthermore, the results suggest some *L. monocytogenes* PFGE types may be adapted to specific niches that facilitate their diffusion with high prevalence in Sweden.

**Table 6.** Number of PFGE types and the six most common PFGE types of human *Listeria monocytogenes* isolates in Sweden, per county, during 1958-2010.

Counties	Population 2011* (%)	Isolates (n)	PFGE- types (N=183)		The most common PFGE types											
			(n)	%	Serovar 1/2a				Serovar 4b				Serovar 4b			
					A:1/2a:4A	%	A:1/2a:4B	%	E:1/2a:9B	%	I:4b:3	%	I:4b:6	%	J:4b:5	%
Skåne	1,252,933 (13.2)	225	79	43.1	9	15.5	15	37.5	8	22.2	17	25.3	25	37.3	14	21.8
Blekinge	152,979 (1.6)	13	14	13	7.1	1	1.7	1	2.5	0	0.0	0.0	1	1.5	1	1.6
Halland	301,724 (3.2)	30	32	20	11.0	3	5.2	3	7.5	2	5.5	0	0.0	2	3.0	2
Västra Götaland	1,590,604 (16.8)	173	18.6	72	39.3	11	19.0	3	7.5	5	13.9	21	31.3	18	26.8	16
Gotland	57,308 (0.6)	8	0.9	6	3.3	2	3.4	0	0.0	0	0.0	1	1.5	0	0.0	1
Kronoberg	184,654 (1.9)	17	1.8	10	5.5	0	0.0	4	10.0	0	0.0	1	1.5	0	0.0	1
Kalmar	233,090 (2.5)	19	2.1	15	8.2	1	1.7	1	2.5	1	2.8	0	0.0	0	0.0	3
Jönköping	337,896 (3.6)	14	1.5	12	6.6	2	3.4	0	0.0	1	2.8	0	0.0	0	0.0	1
Östergötland	431,075 (4.5)	27	2.9	21	11.5	1	1.7	0	0.0	0	0.0	4	6.0	0	0.0	0
Örebro	281,572 (3.0)	12	1.3	9	5.0	0	0.0	3	7.5	0	0.0	1	1.5	1	1.5	1
Södermanland	272,563 (2.9)	13	1.4	11	6.0	0	0.0	2	5.0	2	5.5	0	0.0	1	1.5	1
Västmanland	254,257 (2.7)	34	3.6	26	14.2	0	0.0	1	2.5	1	2.8	4	6.0	3	4.5	2
Uppsala	338,630 (3.6)	32	3.4	24	13.1	1	1.7	1	2.5	1	2.8	0	0.0	0	0.0	5
Stockholm	2,091,473 (22.1)	141	15.1	64	35.0	9	15.5	2	5.0	5	13.9	7	10.4	9	13.4	9
Värmland	272,736 (2.9)	26	2.8	12	6.6	2	3.4	0	0.0	0	0.0	2	3.0	3	4.5	0
Dalarna	276,565 (2.9)	36	4.0	26	14.2	2	3.4	1	2.5	2	5.5	4	6.0	3	4.5	0
Gävleborg	276,130 (2.9)	17	1.8	17	9.3	1	1.7	1	2.5	0	0.0	1	1.5	0	0.0	1
Västernorrland	242,155 (2.5)	56	6.0	29	15.9	8	14.2	2	5.0	7	19.4	0	0.0	0	0.0	4
Jämtland	126,299 (1.3)	4	0.4	4	2.2	1	1.7	0	0.0	0	0.0	0	0.0	0	0.0	0
Västerbotten	259,667 (2.7)	16	1.7	11	6.0	1	1.7	0	0.0	1	2.8	3	4.5	1	1.5	1
Norrbottnen	248,545 (2.6)	15	1.6	9	5.0	3	5.2	0	0.0	0	0.0	1	1.5	0	0.0	0
no data	0	4	0.4	4	2.2	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	1
Total	9,482,855 (100.0)	932	100.0	183	100.0	58	100.0	40	100.0	36	100.0	67	100.0	67	100.0	64

\*www.scb.se

Generally, *L. monocytogenes* isolates collected during 1958–2010 clustered into two lineages, I (serovar 1/2b, 3b, 4b) and II (serovars 1/2a, 1/2c). In lineage I, two groups were prevalent. PFGE group I (193 isolates, serovar 4b) was the largest PFGE group among human isolates that included *L. monocytogenes* isolates identified during the period 1965–2010, and was distributed across all counties. PFGE group J (95 isolates, serovar 4b) was isolated from 1958 to 2008 and identified in 17 counties, mostly in Skåne (25 cases) and Västra Götaland (20 cases). PFGE group D (41 isolates, serovar 1/2b) was present during the entire study period (1958–2010) and was predominant in Skåne (18 cases), but identified in 10 counties. In lineage II, two groups were present during the entire study period: PFGE group E (159 isolates, all serovar 1/2a, except 17 1/2c isolates) was distributed in 19 counties and was the most frequent lineage II PFGE group, followed by PFGE group A (135 isolates, serovar 1/2a) distributed in 20 counties. PFGE group A was observed every year from 1987 and at high frequency (1–20 cases per year). PFGE group B (57 isolates, serovar 1/2a) was first identified in 1963 and remained present to the end of the study period, and was detected in 14 counties but rarely in Norrland region (Table 7; Tables 3 in Papers II and III). These observations suggested few PFGE groups were frequently associated with human listeriosis in Sweden, because of their high prevalence in sources or specific for a single source.

**Table 7.** Number of PFGE groups and the six most common PFGE groups of human *Listeria monocytogenes* isolates in Sweden, per county, during 1958-2010.

Counties	Population 2011 <sup>Δ</sup> (%)	Isolates (n)	PFGE- groups (N=38)		The most common PFGE groups												
					Lineage I						Lineage II						
			%	(n)	%	D	%	I	%	J	%	A	%	B	%	E	%
Skåne	1,252,933 (13.2)	225	24.1	17	44.7	18	43.9	52	26.94	25	26.32	37	27.41	12	21.05	33	20.75
Blekinge	152,979 (1.6)	13	1.4	10	26.3	0	0.0	1	0.52	1	1.05	2	1.15	0	0.0	1	0.63
Halland	301,724 (3.2)	30	3.2	10	26.3	0	0.0	4	2.07	2	2.11	8	5.92	3	5.26	8	5.03
Västra Götaland	1,590,604 (16.8)	173	18.6	27	71.0	5	12.2	46	23.83	20	21.05	20	14.8	10	17.54	23	14.46
Gotland	57,308 (0.6)	8	0.9	6	15.8	0	0.0	1	0.52	1	1.05	2	1.15	1	1.75	0	0.0
Kronoberg	184,854 (1.9)	17	1.8	7	18.4	0	0.0	5	2.59	1	1.05	4	2.96	1	1.75	4	2.52
Kalmar	233,090 (2.5)	19	2.1	8	21.0	1	2.4	2	1.03	3	3.16	2	1.15	1	1.75	8	5.03
Jönköping	337,896 (3.6)	14	1.5	10	26.3	0	0.0	1	0.52	1	1.05	2	1.15	2	3.51	2	1.26
Östergötland	431,075 (4.5)	27	2.9	11	28.9	5	12.2	7	3.62	1	1.05	3	2.22	2	3.51	3	1.89
Örebro	281,572 (3.0)	12	1.3	6	15.8	1	2.4	4	2.07	1	1.05	3	2.22	0	0.0	2	1.26
Södermanland	272,563 (2.9)	13	1.4	8	21.0	0	0.0	2	1.03	1	1.05	3	2.22	0	0.0	3	1.89
Västmanland	254,257 (2.7)	34	3.6	11	28.9	2	4.9	9	4.66	3	3.16	1	0.74	3	5.26	6	3.77
Uppsala	338,630 (3.6)	32	3.4	14	36.8	2	4.9	2	1.03	6	6.32	3	2.22	0	0.0	9	5.66
Stockholm	2,091,473 (22.1)	141	15.1	22	57.9	3	7.3	24	12.43	17	17.9	15	11.1	11	19.3	22	13.84
Värmland	272,736 (2.9)	26	2.8	7	18.4	0	0.0	7	3.62	0	0.0	2	1.15	1	1.75	6	3.77
Dalarna	276,565 (2.9)	36	4.0	12	31.6	2	4.9	11	5.69	0	0.0	3	2.22	5	8.8	5	3.14
Gävleborg	276,130 (2.9)	17	1.8	11	28.9	0	0.0	1	0.52	2	2.11	3	2.22	1	1.75	4	2.52
Västernorrland	242,155 (2.5)	56	6.0	12	31.6	0	0.0	4	2.07	6	6.32	16	11.85	4	7.01	17	10.69
Jämtland	126,299 (1.3)	4	0.4	4	10.5	0	0.0	1	0.52	0	0.0	1	0.74	0	0.0	1	0.63
Västerbotten	259,667 (2.7)	16	1.7	8	21.0	2	4.9	4	2.07	1	1.05	0	0.0	0	0.0	2	1.26
Norrbotten	248,545 (2.6)	15	1.6	7	18.4	0	0.0	5	2.59	0	0.0	5	3.7	0	0.0	0	0.0
no data	0	4	0.4	0	0	0	0.0	0	0.0	3	3.16	0	0.0	0	0.0	0	0.0
Total	9,482,855 (100.0)	932	100.0	38**	0.0	41*	100.0	193*	100.0	95*	100.0	135*	100.0	57*	100.0	159*	100.0

www.scb.se. <sup>Δ</sup>Number of isolates. <sup>\*\*</sup>Number of PFGE groups

<sup>a</sup>www.scb.se. \*Number of isolates. \*\*Number of PFGE groups

Lukinmaa *et al.* (2003) compared 314 human isolates collected during 11 years and found several closely related PFGE types, which are further grouped into major clusters. The PFGE types within a cluster differ in three or fewer fragments and these clusters of closely related PFGE types have been present every year since the 1990s. According to the dendrogram (Lukinmaa *et al.*, 2003), the PFGE types are similar in the region of the small fragments within each cluster. All these clusters have been identified among PFGE groups in Swedish human strains (Papers II and III). A surveillance study of human listeriosis between 1985 and 2014 in Belgium reports that among *L. monocytogenes* serovar 1/2a isolates, the most frequent human pulsovar belong to ST8 and are the only type isolated every year: this type is related to CC8 strains that caused outbreaks in Switzerland, Denmark, Germany and Canada (Bertrand *et al.*, 2016).

In another study (Michelon *et al.*, 2015), three different PFGE standard operating procedures (SOP) were compared and all PFGE profiles gained were similar in quality and indistinguishable except one profile. Although PFGE profiles "... are both similar and comparable..." (Michelon *et al.*, 2015), the surveillance system needs to be improved to be able to compare PFGE profiles from both human and food isolates (Michelon *et al.*, 2015). However, the European Union Reference Laboratory for *L. monocytogenes* (EURL for *Lm*) that uses PFGE profile interpretation, according to SOP, does not recommend the analysis of profiles below the 33 kb limit (Felix *et al.*, 2013). This omits an important part of the bacterial genome, and although small fragments below 33.3 kb were non-detectable in *L. monocytogenes* lineage I isolates, those small fragments were present in all lineage II isolates (Papers II and III). Thus, small bands can be used to differentiate between lineage I and lineage II strains. Several sequenced *L. monocytogenes* strains belonging to both lineages analysed *in silico* with PFGE and *AscI* corroborated this observation (Papers II and III). With *in silico* analysis, it is possible to detect the presence of a small fragment (1454 bp) in all *L. monocytogenes* lineage I strains. Although whole genome sequencing is an increasingly used subtyping method, it is not accessible in all microbiological laboratories (Camargo *et al.*, 2016; Kramarenko *et al.*, 2016).

### *PFGE types found in Swedish studies*

A retrospective characterisation of *L. monocytogenes* strains isolated in published and unpublished Swedish studies from outbreaks and some sporadic cases are described below, along with *L. monocytogenes* PFGE types from foodstuffs analysed in previously published and unpublished Swedish studies. This is done in order to put PFGE types, found in Sweden during the 53 year period, into a broader context. The PFGE type nomenclature is according to the type designation formulated in Papers II, III and section 4.4.4 in this summary (Analysis of PFGE profiles).

Data from the retrospective studies (Papers I, II and III) indicated five *L. monocytogenes* isolates of patients in the suspected outbreak in 1981 (reported by Bernander in 1983 and described in Section 2.8.2) belonged to different lineages, PFGE groups, and PFGE types. These were lineage I PFGE types I:4b:3 (an adult and an infant case), I:4b:1, J:4b:5 and lineage II PFGE type K:1/2a:134. At least three cases shared the same PFGE group (I), and the PFGE types (I:4b:1 and I:4b:3) were closely related and probably part of a local outbreak. These two types were isolated

from 23 and 67 human cases in the study period. PFGE type J:4b:5 belonged to PFGE group J, the fourth largest PFGE group with 95 isolates/cases (Paper II), where the first case was identified in 1958, and included 9 PFGE types. Bernander (1983) found all clinical isolates in the suspected outbreak belong to 4b, however, one of the human isolates was identified as serovar 1/2a (PFGE type K:1/2a:134), thus, the sample may have contained two different strains (Tham *et al.*, 2002; Paper III). PFGE type J:4b:5 was isolated from two fish products in 2002 and from a milk product in 1991. PFGE group K is the seventh most common PFGE group in the period studied, with 34 human cases, and included 10 PFGE types. The PFGE group K was identified in the early 1980s (Paper III).

The first documented food-borne case of listeriosis in Sweden occurred in 1993 and was due to medwurst (Loncarevic *et al.*, 1997). The *L. monocytogenes* strain was identified as PFGE type E:1/2a:31, and this type was involved in 17 cases during the study period (1958–2010): 8 cases from 1984 to 1992 and 9 cases between 1993 and 2010. This PFGE type belonged to lineage II, PFGE group E, and was the second largest PFGE group among human isolates (159 cases), with a distribution into 28 PFGE types (Paper III).

The rainbow trout outbreak occurring in Värmland in 1994–1995 (Ericsson *et al.*, 1997) involved PFGE types Q:4b:10 (six cases), I:4b:3 (one case) and I:4b:16 (one case). The ninth human isolate shared PFGE type I:4b:6 and was not considered part of the outbreak (Papers I and II). The four *AscI* PFGE types belonged to lineage I, and serovar 4b and three PFGE types belonged to PFGE group I and one to PFGE group Q. PFGE type Q: 4b:10 was isolated from 12 human cases between 1974 and 1995, including eight human cases during the rainbow trout outbreak (1994–1995). This type was the most common within PFGE group Q and included six PFGE types (Paper II). The PFGE type I:4b:6 was isolated from three fish products (two products in 1993 and one product in 1998).

A sporadic case of gastroenteritis occurred in 1993 was due to consumption of gravad salmon from brand X. The *L. monocytogenes* isolates serovar 4b from human patient faeces sample and a salmon sample shared identical PFGE patterns with *Apal* and *SmaI* enzymes. This PFGE type was found previously in a Swedish salmon plant (brand X) and was identical to the French epidemic type causing an outbreak due to consumption of pig tongue in aspic in 1992–1993 (Personal communication: Jacquet, 1994). The PFGE type found in both the pig tongue in aspic and in salmon samples was I:4b:6. However, this long Swiss epidemic phagetype is the epidemic cheese-borne strain from Swiss, Californian, Danish and French outbreaks during the 1980s and 1990s (Tham *et al.*, 2000; Tompkin *et al.*, 2002). In Japan in 1991, serovar 4b was predominant (60%) among human *L. monocytogenes* isolates, whereas, serovar 1/2b only accounted for 30%. The long Swiss epidemic phagetype was identified among 102 Japanese *L. monocytogenes* isolates (82 human isolates from 1978–1993 and 20 isolates from food sold in Japan 1989–1993). With PFGE and three restriction enzymes, one isolate from raw sausage and 17 of 82 human Japanese *L. monocytogenes* isolates were found to be indistinguishable from the long Swiss epidemic phagetype (Nakama *et al.*, 1998). Of 107 *L. monocytogenes* human isolates collected in Sweden during 1976–1985, 47 shared the long Swiss epidemic phagetype. Of those, 19 isolates showed the same characteristics (restriction with *Apal*, *SmaI* and *XhoI*) as the epidemic strains from Switzerland (1983–1987) and Denmark (1985–1987 & 1989–1990; Ericsson *et al.*, 1996). Although, this indistinguishable PFGE type (I:4b:6) was identified in



different sources globally, it appears cheeses are the main source as large cheese outbreaks due to this epidemic type were reported from several countries during the 1980s and 1990s.

In the two sporadic cases associated with cheese and Chinese cabbage in 1998 in Västernorrland (Tham *et al.*, 1998, Section 2.8.2), the *AscI* PFGE type A:1/2a:4A was found in all samples from patients and food. This type corresponds to the *AscI* PFGE type 0038 (EFSA nomenclature) and ST7. Type A:1/2a:4A was the most common lineage II PFGE type in the study period. The PFGE group A was the third most common and included 17 PFGE types (Papers I and III). In Finland, PFGE “type 1” closely related to the Swedish type A:1/2a:4A (unpublished data) and has been common in sporadic cases from 1994 onwards (Lukinmaa *et al.*, 2003). PFGE type A:1/2a:4A has been isolated from RTE fish products between 2000 and 2010 (Netterby, 2000).

The *AscI* PFGE type encountered in all samples from the non-invasive outbreak in Hälsingland (2001) was A:1/2a:4B (Carrique-Mas *et al.*, 2003; Danielsson -Tham *et al.*, 2004). Moreover, the epidemic strain from the meat-borne outbreak in 2013–2014 in Sweden, involving 49 human cases, belonged to the *AscI* PFGE type 0039 and ST7 (Public Health Agency of Sweden, 2015 and 2016; Dahl *et al.*, 2017) and was identical to PFGE type A:1/2a:4B. This PFGE type, with MLVA profile 07-07-09-10-06, is also regularly isolated from clinical cases in Norway (30%) and has been involved in two listeriosis outbreaks in Norway: from a meat slicer at Ålesunds hospital in 2005 and from ecological camembert cheese at Rikshospitalet in 2007 (Lindstedt *et al.*, 2008; Johnsen *et al.*, 2010; Lunestad *et al.*, 2013; Personal communication: Lin Thorstensen Brandal, 2017; Paper I). The PFGE type A:1/2a:4B was isolated from cheese in 2001–2002 and from RTE fish products in 2000–2002 (Mandorf, 2003). The two closely related PFGE types (A:1/2a:4A and A:1/2a:4B) belong to PFGE group A and ST7. The PFGE type A:1/2a:6 belongs to PFGE group A, and to ST19, has been implicated in the Swedish fish-borne outbreak between 2013 and 2015 (Public Health Agency of Sweden, 2015 and 2016). This PFGE type (A:1/2a:6) was isolated from five human cases in the study period (1958–2010), the first case in 1995 and then four cases during 2000–2010 (Paper III). According to MLST analysis, among these five *L. monocytogenes* isolates, four belong to ST19 and one to ST7 (Personal communication: Hedenström, 2017).

In 2000–2002, two research projects analysed the prevalence of *L. monocytogenes* in vacuum-packed gravad and cold-smoked salmon/rainbow trout (Section 2.9.3). In the first study (Netterby, 2000), the sample with the largest cfu/g harboured three PFGE types (A:1/2a:4A, B:1/2a:3A and G:1/2b:2A) and these types were found in two different plants and were common among human isolates in the same year. An additional PFGE type (K:1/2A:25) was found in one fish processing plant (Netterby, 2000). In the second study, Mandorf (2003) analysed 40 fish products from nine fish plants: four fish plants were contaminated with the pathogen. All isolates belonged to serovar 1/2a distributed into eight different *AscI* PFGE types (all identified among human clinical isolates). In six fish samples from plant A, the *AscI* PFGE type E:1/2a:9A was identified (see below). One gravad salmon sample from plant E harboured three PFGE types belonging to two different PFGE groups (B:1/2a:114, B:1/2a:125 and F:1/2a:13) and one cold-smoked salmon sample from plant A harboured two PFGE types (E:1/2a:9A and E:1/2a:12A). The PFGE type G:1/2b:2A was involved in 19 human cases during the study period. This PFGE type belonged to lineage I, PFGE group G, and was the tenth most common PFGE group among human isolates (27 cases) and was distributed into 5 PFGE types (Paper II).

The PFGE type B:1/2a:3A was isolated during 1996–2003 in six human cases and the closely related PFGE type B:1/2a:3B was identified in 1981, and until 2007, among seven cases. In 2000, both types were involved in three (B:1/2a:3A) and four human cases (B:1/2a:3B: Paper III). The PFGE type B:1/2a:3A was isolated from RTE fish products in 2000–2001 and the B:1/2a:3B type was isolated from RTE fish products in 2001 and from meat products (hot dogs) in 2011 (Netterby, 2000). PFGE type B:1/2a:22 was isolated from humans only once in 2001 (Paper III), although this type was isolated from RTE fish products between 1989–2002 and from soft cheeses in 1988 and 1993 (Loncarevic *et al.*, 1998a; Netterby, 2000; Mandorf, 2003). The PFGE type B:1/2a:114 was isolated from only 4 human cases in the study period, even though it was first identified in 1972 and reappear in 2006–2007 with three isolates/cases (Paper III), but was isolated from fish products between 2000–2010 and Gorgonzola cheese in 2001 (Netterby, 2000; Mandorf, 2003). The PFGE type B:1/2a:125, belonging to the same PFGE group (B), was identified in one human case in 1963 (Paper III) and in RTE fish products during 2000–2002 (Mandorf, 2003). All these PFGE types belonged to PFGE group B, that is the fifth most common PFGE group, with 57 cases and included the largest number of PFGE types (26) (Paper III).

The PFGE type F:1/2a:13 was involved in 6 cases between 1988 and 2006 and belonged to lineage II, PFGE group F, and was the eleventh most common PFGE group with 26 cases and was distributed into 8 PFGE types (Paper III). This type was isolated from RTE fish products during 1988–2002 (Loncarevic *et al.*, 1998a; Netterby, 2000). The PFGE type E:1/2:12A included two serovars (1/2a and 1/2c) was first isolated in 1962, and was isolated from 15 human cases up to 2006. PFGE type E:1/2:12A was closely related to PFGE type E:1/2:12B which also shared two serovars but was found in only six human cases between 1989 and 2005 (Paper III). PFGE type E:1/2:12A was identified in RTE fish products in 2001 and in 2011 and in meat products in 1993–1994 (Netterby, 2000). The PFGE type K:1/2a:17 was first identified in two human cases in 1981 and was involved in 11 human cases during the study period, with five cases in 1988–1989 and three cases in 2000–2001 (Paper III). This PFGE type was identified in fish products between 1988 and 2002 (Loncarevic *et al.*, 1998a ; Netterby, 2000; Mandorf, 2003). PFGE type K:1/2A:25 shares the same PFGE group as K:1/2A:17 and has been only seen in three human cases: it was isolated from RTE fish products in 2000–2001 (Netterby, 2000). These two PFGE types belonged to lineage II and PFGE group K was the seventh most common PFGE group among human isolates (34 cases) and was distributed into 10 PFGE types (Paper III).

The PFGE type E:1/2a:9A was seen in 20 human cases (1994–2010), and was the seventh most common type, with 14 cases in 2002–2003. The closely related PFGE type E:1/2a:9B was isolated in 36 human cases (1993–2010) and involved in 30 human cases between 2003 and 2010 (Papers I and III). Both PFGE types were isolated from RTE fish products, the PFGE type E:1/2a:9A, in 2002, and the PFGE type E:1/2a:9B, in 2001, 2002 and 2010 (Mandorf, 2003). These two PFGE types corresponded to the most common human pulsotypes in Denmark, *AscI* 40-*ApaI* 42, PFGE type E:1/2a:9A (82 isolates) and *AscI* 38-*ApaI* 48, PFGE type E:1/2a:9B (40 isolates), and were isolated from human cases in Denmark between 2002 and 2012. These closely related pulsotypes differ by only two bands in the *AscI* profiles and one band in the *ApaI* pattern (Kvistholm Jensen, 2016). In Finland, an increase in listeriosis in 2010 was partly caused

by a specific *AscI* type, *Lm96*, which caused 19% of human cases during 2010 and was the most prevalent type found in food isolates in Finland in 2010. The type *Lm96* was identical to the Danish *AscI* profile 38, the second most common *AscI* profile belonging to CC8 in Denmark, and was found in a specific fishery production plant in Finland and in cold-smoked rainbow trout products from the plant (Nakari *et al.*, 2014, Kvistholm Jensen, 2016). Consequently, the Swedish *AscI* PFGE type E:1/2a:9B, the Danish 38-48, and the Finnish *Lm96* have identical *AscI* profiles.

Among *L. monocytogenes* isolates found in cold-smoked salmon and gravad salmon packed under modified or vacuum atmosphere, five *AscI* PFGE types were identified (Paper IV). Three PFGE types were isolated from manufacturer A (B:1/2a:114, E:1/2a:49 and Q:4b:2), whereas, PFGE types E:1/2a:93 and F:1/2a:48 were from different manufacturers. The PFGE type E:1/2a:49 was first identified in a human patient in 1985 and was implicated in 18 human cases during the study period (1958–2010), with 4 cases in 2007 and 12 cases during 2009–2010 (Paper V). This PFGE type was isolated from RTE fish products in 2005 and 2010 (Paper IV). The PFGE type E:1/2a:93 was only seen in four human cases during 1985–2009 (Paper III) and was isolated from fish products in 2001 and 2005 (Paper IV). The PFGE type F:1/2a:48 was implicated in eight human cases during 2003–2010 (Paper III).

## 5.6 Relationship between PFGE group, PFGE type and serovar

All *L. monocytogenes* isolates within a given PFGE group in both lineages I and II shared the same serovar *i.e.* PFGE groups were homogeneous with respect to serovar, except lineage I PFGE group G and lineage II PFGE group E, which both included two serovars. In lineage I, five PFGE types belonged to PFGE group G sharing serovar 1/2b and only one PFGE type sharing serovar 3b, G:3b:2B (Paper II). Revazishvili *et al.* (2004) also identified isolates with the same PFGE type that sometimes belonged to different serovars (3b and 1/2b). However, in lineage II, the majority of isolates belonging to PFGE group E were serovar 1/2a. Four PFGE types were distributed on either serovar 1/2a or 1/2c. These were PFGE types E:1/2a:12A (6 isolates) and E:1/2c:12A (9 isolates); E:1/2a:12B (4 isolates) and E:1/2c:12B (2 isolates); E:1/2a:93 (2 isolates) and E:1/2c: 93 (2 isolates); and, E:1/2a:105 (1 isolate) and E:1/2c:105 (1 isolate). Furthermore, PFGE group E also included two PFGE types sharing only serovar 1/2c, E:1/2c:36 (2 isolates) and E:1/2c:95 (1 isolate). However, PFGE group N (lineage II) included all three PFGE types of serovar 1/2c, indicating this group was more genetically homogeneous (Paper III). Gianfranceschi *et al.* (2009) report nearly complete correlation between pulsotypes and serotypes: only 5% of all isolates with the same pulsotype belonged to different serotypes. Lukinmaa *et al.* (2003) report a few *L. monocytogenes* isolates with indistinguishable PFGE profiles displaying different serotypes (1/2a and 3a). These data suggest PFGE groups provide adequate discrimination between serotypes.

Although the *L. monocytogenes* isolates were from different sources, time and location, the diversity of PFGE types within PFGE groups and homogeneity of PFGE groups among PFGE types were observed during fifty-three years (1958–2010) in Sweden. Seventeen (44.7%) of 38 PFGE groups were each represented by only one PFGE type, six groups by two types and two groups by three types. Three *L. monocytogenes* PFGE groups contained higher numbers of PFGE

types among human isolates than other PFGE groups during the study period: PFGE group B, serovar 1/2a (26 PFGE types), PFGE group A, serovar 1/2a (17 PFGE types) and PFGE group D, serovar 1/2b (15 PFGE types) (Papers II and III), suggesting these small bands have high conservation within PFGE groups. Similarly, Cantinelli *et al.* (2013) observe the homogeneity of MvLST gene sequences of *L. monocytogenes* within clonal complexes (CCs) among isolates collected from 13 countries in Europe, America and Asia between 1963 and 2000. Numerous PFGE types have been involved in large national and multinational outbreaks and these types, called epidemic clones (EC), have been defined based on MEE, ribotyping and PFGE, and later by MvLST. Some representative epidemic clones such as ECI and ECII, both belonging to serovar 4b, and ECIII, belonging to serovar 1/2a, have been included in major groups through MLST and are called Clonal Complex (CC), *e.g.*, ECI was included in CC1, ECII in CC6, and ECIII in CC11 (Cantinelli *et al.*, 2013). Most CC of *L. monocytogenes* have persisted over decades and only a small number of new CC have been identified in both lineages I and II (Haase *et al.*, 2014). Similarly, few new PFGE groups have appeared among human isolates in Sweden during the period 1958 to 2010. A limited number of PFGE groups have increased in frequency over decades and have been responsible for several cases of human listeriosis in Sweden, possibly due to some *L. monocytogenes* strains persisting during long periods in food processing plants and continuously contaminating RTE foods.

The recombination rate of *L. monocytogenes* is one of the lowest in bacterial species (Ragon *et al.*, 2008). Among MLST genes, recombination has only been identified at gene *dapE* in lineages I and II. In MvLST genes, recombination in lineage II has been identified at genes *actA*, *intA* and *dal*, whereas, in lineage I, recombination has only been distinguished at *inlB* locus. Moreover, the effect of recombination is higher in lineage II than in lineage I (den Bakker *et al.*, 2008; Cantinelli *et al.*, 2013). At the same time, lineage I is more affected by positive selection, which occurs among MvLST genes *actA* and *inlC* and MLST loci *dapE* and *ldh* (Cantinelli *et al.*, 2013). These observations confirmed housekeeping genes are more conservative than virulence genes (Nightingale *et al.*, 2005; den Bakker *et al.*, 2010; Nightingale, 2010). However, there was more of a difference in the average number of isolates between lineages I and II based on PFGE types (Papers II and III). In lineage I, the average number of isolates was 6.8 isolates per PFGE type, and in lineage II, the average number of isolates was 4.2. In contrast, the average difference was low, based on the PFGE group. In lineage I, the average number of isolates was 25 and in lineage II, the average number of isolates per PFGE group was 24 (Papers II and III). The *L. monocytogenes* genome is highly conserved (Kuenne *et al.*, 2013); although, within *L. monocytogenes* clonal groups, there is some degree of diversity at whole genome level (Chen *et al.*, 2016a).

## 5.7 PFGE and *L. monocytogenes* genetic variants

Burn (1935) described the occurrence of three *L. monocytogenes* variants in cultures from animal organs and cultures that had been transferred several times in immune serum (Burn, 1935). One of the strains lost the property of haemolysing blood in agar and broth media, but the other biochemical, morphological and serological characteristics remained unchanged. The second variant lost its morphological characteristics in broth medium, but not the property of haemolysing blood. The third variant appeared in meat infusion broth after six to eight weeks

and although the biochemical characteristics remained unchanged, the morphological characteristics differed from the original strain (Burn, 1935).

Several studies cover cases and outbreaks associated with two or more closely related *L. monocytogenes* genetic variants, where the *AscI* PFGE profiles usually differ by only two bands above 250 kb and are similar in the region below 145.5 kb (Kathariou *et al.*, 2006; Gilmour *et al.*, 2010; Lomonaco, *et al.*, 2013; Tham *et al.*, 2013; Kvistholm Jensen, *et al.*, 2016). In the early 1990s, Austrian and French researchers considered “such differences in profiles might be due to recent mutations during the outbreak, during isolation procedures or during storage of the cultures, or might in contrast be due to sporadic cases superimposed on the epidemic” (Buchrieser *et al.*, 1993). Two closely related *L. monocytogenes* strains were isolated from blood samples from two human cases (Paper V). In the first case, both strains belonged to PFGE group E but shared different PFGE types. Similarly, in the second case, the two strains belonged to the same PFGE group F, but shared different PFGE types, *i.e.* DNA pattern above 145.5 kb.

In the USA, two outbreaks of listeriosis were associated with RTE meat products with hot dogs (1998–1999) and turkey deli meats (2002) being implicated. The *AscI* PFGE profiles from the two outbreaks were closely related and belonged to serovar 4b, ECII, MvLST 19, ST6 and CC6 (Kathariou *et al.*, 2006; Chen *et al.*, 2007; Chen *et al.*, 2016a ). The PFGE profiles differed in the largest bands, but not in the small bands below 145.5 (Kathariou *et al.*, 2006). The differences were detected in prophage and internalin-like gene sequences produced by a single point mutation. In a French study (Charlier *et al.*, 2012), three 4b *L. monocytogenes* strains were identified in the same synovial fluid from a joint sample. Analysis with PFGE and *AscI* restriction enzyme identified three PFGE types, whereas, MLST analysis identified two sequence types (ST): two strains belonged to ST160 and the third to ST161. The difference in ST was related to a single mutation in the amplified *ldh* gene fragment. In the two strains belonging to the same ST160, the strains were identical in the *AscI* PFGE profiles below 145.5 kb, but differed by three bands above 145.5 kb (Charlier *et al.* (2012) assume “these three isolates derive from a unique clone but have likely evolved within the patient’s infected joint”. The two closely related strains in the studies by Kathariou *et al.* (2006) and Charlier *et al.*, (2012) appear identical in the *AscI* restricted fragments < 145.5 kb *i.e.*, the isolates belonged to the same PFGE group.

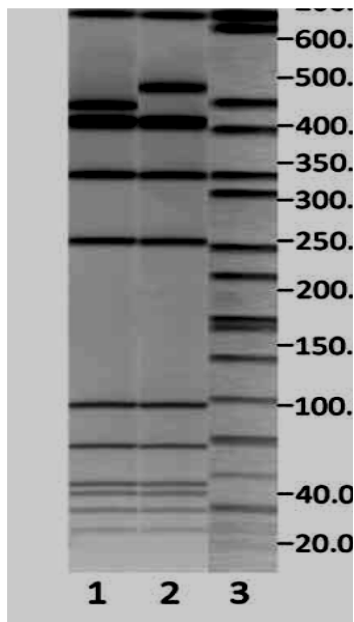
In Finland, during six years, 37 *L. monocytogenes* isolates were collected from an ice cream processing environment and equipment and 11 serovar 1/2b PFGE types were identified with three different restriction enzymes: PFGE type II was predominant (26 isolates) and was even found in the ice cream. PFGE type II persisted during six years, whereas, the other 10 types “...were single findings or appeared twice during the same year...” and appear to be a “...mutant of the PFGE type II, since most of them differed only by one or two bands...” (Miettinen *et al.*, 1999a). Similarly, in the USA, several *L. monocytogenes* strains were detected with closely related *AscI* profiles belonging to serovar 1/2b or 3b, CC5 and ST5 in contaminated ice cream facilities, which caused an outbreak in 2010–2015. SNP-based subtyping identified two clusters and each cluster had two clades. The two clusters identified were consistent with *AscI* PFGE groups, but each PFGE group contained three *AscI* PFGE types. In clades Ia and IIa, *AscI* PFGE also identified one *AscI* PFGE profile in each clade, but in clades Ib and IIb, *AscI* PFGE identified two *AscI* PFGE profiles in each clade. Isolates of serovar 1/2b cluster I differed in the

presence or absence of three intact prophages, which were visible among *AscI* PFGE profiles (Chen *et al.*, 2017b). In another Finnish study, the occurrence of *L. monocytogenes* in RTE fish products was studied by Johansson *et al.* (1999) during 14 months. With the use of two restriction enzymes, nine PFGE types serovar 1/2a were identified in a fish processing plant, however, only four types of serovar 1/2a were identified in the fish products from retail markets and two were not found in the plant. PFGE type A was dominant among the isolates and was persistent, whereas, the other types were found sporadically. These types “...differed by only 1–2 bands indicating a close relationship of the strains” (Johansson *et al.*, 1999). Although various PFGE types were detected in an outbreak involving a manufacturer, both *AscI* PFGE and WGS were consistent to cluster related clonal groups.

Two closely related *AscI* PFGE types (38 and 40), were identified among 122 human clinical cases collected between 2002 and 2012 in Denmark. Both PFGE types belonged to serovar 1/2a, CC8 and ST8. (Kvistholm Jensen *et al.*, 2016). These two PFGE types corresponded to the Swedish PFGE types E:1/2a:9A and E:1/2a:9B, which were isolated in 56 human cases between 1993 and 2010 (Paper I and III). The two *AscI* profiles differed by only two bands above 400 kb (Fig. 1). In Canada in 2008, two closely related *AscI* PFGE patterns (08-5578 and 08-5923), serovar 1/2a, caused a large outbreak due to *L. monocytogenes* contaminated RTE meat products. These two similar but distinct PFGE types were collinear, harboured a 50 kbp putative mobile genomic island encoding translocation, resistance and regulatory determinants, but differed by 28 SNPs and three insertion/deletions. The differences in *AscI* restriction patterns were two bands above 400 kb that were due to the presence of a 33 kb prophage in profile 08-5578 (Gilmour *et al.*, 2010). The two closely related strains in both the Danish and Canadian studies belonged to serovar 1/2a and CC8 (Gilmour *et al.*, 2010; Kvistholm Jensen *et al.*, 2016), and appear identical in the *AscI* restricted fragments <145.5 kb *i.e.*, the isolates are considered to belong to the same PFGE group. Multiple closely related PFGE types among *L. monocytogenes* isolates sharing similar PFGE groups have been observed, where WGS confirmed they evolved from a common ancestor albeit prophage diversification in the genome was identified (Chen *et al.*, 2017b).

Generally, throughout Sweden, only one isolate of *L. monocytogenes* from patients with invasive listeriosis is submitted by clinical laboratories to the Public Health Agency. As more than one serovar or PFGE type can be isolated from a single sample (*i.e.* clinical, animal and food), this complicates the epidemiological investigation of the sources and routes of *Listeria* infection (Loncarevic *et al.*, 1996a; Tham *et al.*, 1999; Paper V). Three gravad salmon samples purchased in Sweden, harboured two PFGE types each from different lineages (E:1/2a:49 and Q:4b:2: Paper IV). In a mother-baby pair, Seeliger (1962) identified *L. monocytogenes* serovar 4b in the baby and *L. monocytogenes* serogroup 3 in the mother. Furthermore, in one chinchilla both *L. monocytogenes* serovar 4b and *L. monocytogenes* serogroup 1 were isolated (Seeliger, 1962). Therefore, for investigating sources and pathways of *L. monocytogenes* infection, several isolates from each sample need to be characterised and isolates from humans and food need to be compared in order to minimise misleading and erroneous results. Simultaneously, the discriminatory power of a subtyping method should be carefully evaluated as excessive discrimination can lead to illogical relations between isolates and a genetic relationship cannot be determined (Chen *et al.*, 2017b).

Acquisition or loss of bacteriophages in bacterial genomes changes all genomes, and generates genetic variants. Thus, random genetic events, including point mutations, insertions and deletions of bacterial DNA can alter the PFGE patterns. PFGE analysis with *AscI* is suitable for identifying gain or loss of prophages in *L. monocytogenes* genomes usually located at the larger restriction fragments. Even with differences in SNP results, the same conclusions were reached with PFGE, as strains were related. PFGE with restriction enzyme *AscI* appeared appropriate for grouping PFGE types into PFGE groups and enabled the definition of 932 human *L. monocytogenes* isolates into at least 38 PFGE groups, based on the small bands below 145.5 kb, with 17 groups belonging to lineage I and 21 groups to lineage II.



**Fig.1.** Closely related PFGE types. *AscI* profiles of *L. monocytogenes* lineage II, Clonal Complex 8 and ST8. Lane 1 (PFGE type E:1/2a:9b); Lane 2 (PFGE type E:1/2a:9a); Lane 3 (Salmonella Braenderup H9812). (Kvistholm Jensen *et al.*, 2016). Published with permission by Kvistholm Jensen 2019.

## 6 CONCLUSIONS

1. Generally, in Sweden, the incidence of listeriosis is higher in males and in an aging population (> 60 years), as has been observed in other countries. Pregnancy-associated cases have decreased over time. The primary route of human listeriosis is ingestion of contaminated RTE foods. In several studies around the world, *L. monocytogenes* has been isolated in high frequencies from soft cheeses, hot- or cold-smoked or gravad fish and deli-meat products, and this trend is similar in Sweden. Food contamination occurs through the processing environment and from the raw material to the final product. *L. monocytogenes* can persist over decades in processing plants.
2. The conventional serotyping by agglutination identifies five main *L. monocytogenes* serovars belonging to lineage I (4b, 1/2b and 3b), and lineage II (1/2a and 1/2c). Although, there is no association between serovars, gender and age, the prevalence of serovar 1/2c among males, and serovar 4b in pregnancy cases is reported globally, including Sweden. A shift from *L. monocytogenes* serovar 4b to serovar 1/2a among clinical human *L. monocytogenes* isolates occurred in the mid 1990s and early 2000s in Europe, including Sweden.
3. Pulsed field gel electrophoresis (PFGE) with restriction enzyme *AscI* is efficient in distinguishing *L. monocytogenes* subtypes and variants (based on the number and distribution of all detectable fragments in a DNA restriction profile). Among 932 human isolates, 183 PFGE types were identified, indicating relatively low genetic diversity.
4. Some human *L. monocytogenes* PFGE-types were present over extended periods during the study period (1958–2010). Others were isolated only during limited periods. Six PFGE types belonging to serovar 1/2a and 4b have persisted at least between 17 to 47 years and are frequently associated with human listeriosis in Sweden. The two most common PFGE types are distributed across 16 to 17 counties. Some PFGE types have been identified globally, indicating certain *L. monocytogenes* PFGE types may be adapted to specific niches and are thus widely distributed with a high prevalence.
5. Based on the number and distribution of all detectable small fragments below 145.5 kb, *L. monocytogenes* PFGE types could be further divided into PFGE groups. Although the *L. monocytogenes* isolates are from different sources, time and location, the small fragments below 145.5 kb are genetically more conserved than the larger fragments, supporting the utility of the genomic region of small fragments in grouping *L. monocytogenes*. The distribution of these small fragments establishes the relatedness of strains and defines genetic markers for both lineages I and II. The three most common PFGE groups are distributed across 19, 20 and 21 counties, suggesting specific PFGE groups may be frequently associated with human listeriosis in Sweden. As a new PFGE group rarely appears, it is reasonable to assume the most important PFGE groups of *L. monocytogenes* have been identified in Sweden.
6. *L. monocytogenes* PFGE types are homogeneous with respect to serovar. The genomic region of small fragments below 145.5 kb establishes an association between PFGE



group and serovar. The number of PFGE types is considerably higher in lineage II than in lineage I, whereas, the numbers of PFGE groups in both lineages are equivalent. In contrast to lineage II, *L. monocytogenes* isolates, small DNA fragments below 33.3 kb are nonvisible in all isolates belonging to lineage I. Thus, lineage I isolates and lineage II isolates do not share any PFGE group. In consequence, the small fragments are able to differentiate the lineages and confirm the relatedness of strains.

7. A new procedure for improving the identification of a *L. monocytogenes* isolate is proposed. First, the smallest DNA fragments below 145.5 kb are analysed to identify the *AscI* PFGE group, and then the large fragments above 145.5 kb are used to determine the PFGE type. As the small DNA fragments in the *AscI* PFGE profiles are relatively conservative, they can be used for an initial rough division of profiles. PFGE with *AscI* restriction enzyme is still a valuable method for genomic analysis and comparison for identifying genomic relatedness among *L. monocytogenes* isolates. Consequently, the application of PFGE *in silico* subtyping enhances the WGS analysis through first identifying the PFGE group and then the PFGE type, which allows rapid screening of genomes. However, the genetic structures of the small restriction fragments in *AscI* PFGE groups of *L. monocytogenes* genomes remain to be determined.

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